Transfer of Wild-Type Plasmids Harbouring Tetracycline or Erythromycin Resistance Genes from Native Strains of Lactobacillus plantarum to other Bacteria in a Gastrointestinal Environment

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Transfer of wild-type plasmids harbouring tetracycline or erythromycin resistance genes from native strains of *Lactobacillus plantarum* to other bacteria in a gastrointestinal environment

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Ph.D. Thesis

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Preface

The work presented in this thesis has been performed at Department of Microbiology and Risk Assessment under the National Food Institute, Technical University of Denmark (DTU). The Ph.D.-project was conducted from April 2004 to April 2008, interrupted by maternity leave with my son Sebastian during the third year. The work has formed part of the EU-project ACE-ART (funded by the European Commission grant CT-2003-506214), which was initiated in order to increase the knowledge of antibiotic resistant lactic acid bacteria in the food chain and thus create a better basis for risk assessment of these.

The time working with this project has been exciting and challenging for me. I appreciate both the professional guidance and feed-back as well as the practical help and the pleasant atmosphere that people at the department, in the EU-project and otherwise around me has created during this time.

Particularly, I would like to thank Sigrid R. Andersen who believed in my qualities from the beginning and gave me a good start being my daily-day supervisor at the National Food Institute during the first year. I'm also very grateful to Andrea Wilcks for taking over the assignment and complementing the supervision in the best possible way and besides for being a very sweet personality. My internal supervisor Karin Hammer at Department of Systems Microbiology, BioCentrum-DTU, DTU, has contributed with great engagement and many interesting ideas - it has been a pleasure being your student.

I want to thank the technicians Kate Vibefeldt and Bodil Madsen for their competent work in the laboratory and without whom I would still be plating faeces from animal experiments. The "applied microorganism group" and especially Tine Rask Licht and Jens Bo Andersen are skilled trouble-shooters in microbial ecology and molecular microbiology and have been a great comfort to share roof with.

To my husband Troels, who has run with double speed on the home front – you're wonderful.

Mørkhøj, April 2008

Louise Feld

Content

Summary

This thesis deals with conjugation of plasmids harbouring tetracycline or erythromycin resistance genes from wild-type lactobacilli to other bacteria relevant to the human gut. Conjugal transfer and factors affecting transfer has been evaluated both *in vitro* and in different animal models simulating the human gastrointestinal (GI) tract. The experimental work encompasses aspects trying to elucidate the extent of antibiotic resistance gene dissemination mediated by lactobacilli - both by means of transfer frequency giving an estimate of the quantitative contribution as well as the pathways of transfer, hereunder potential recipient bacteria. An assessment of selective pressure with antibiotics both at sub-inhibitory concentrations and concentrations simulating clinical doses and its effect on distribution of antibiotic resistance plasmids is also included. Additionally, a more mechanistic point of view has been applied with emphasis on plasmid genetic factors amongst others responsible for transfer, determination of host-range, expression of antibiotic resistance and regulation of plasmid replication.

The present thesis consists partly of a theoretical overview incorporating available literature concerning the subject and partly of three enclosed manuscripts accounting for important selected results obtained during the project.

Main areas in the theoretical part are application and action of antibiotics and development of antibiotic resistance, including pathways of dissemination and resistance mechanisms. Furthermore, a paragraph has been devoted to gene transfer in bacteria looking more into the genetic basis of transferable DNA elements and their interaction with bacterial hosts. Lastly, antibiotic resistance gene transfer in the human GI tract and specific models, which can be applied to study this, are discussed. In the theoretical overview, special emphasis has been paid to the antibiotics tetracycline and erythromycin as well as lactobacilli, conjugation and rolling-circle replicating plasmids, since these belong to subjects practically investigated in this project.

In **Paper I**, two wild-type *Lactobacillus plantarum* strains, DG 507 and DG 522, isolated from Belgian fermented sausages and harbouring native transferable plasmids conferring erythromycin and tetracycline resistance were tested for their ability to function as donors in a gastrointestinal environment. Germ-free rats were associated with an *Enterococcus faecalis* recipient and used as a model to simulate a worst-case scenario of transfer in the human GI tract. Following colonization of the recipient, each of the two donor strains was administered to the rats, and from the second day

onwards, faecal samples containing transconjugants were derived from either donor. The study documented the ability of both *Lactobacillus* strains to facilitate conjugation after being subjected to physical factors resembling those encountered in the human GI tract after ingestion.

In **Paper II**, transfer of wild-type plasmid pLFE1 carrying an erythromycin resistance gene was investigated in a di-associated rat model, as described for manuscript 1. First, an *E. faecalis* recipient was allowed colonization in the rats and thereafter a wild-type donor strain *Lactobacillus plantarum* M345, isolated from a French raw-milk cheese and carrying pLFE1, was introduced. Dissemination of pLFE1 was examined in three groups of rats receiving either erythromycin at concentrations simulating clinical doses, 1/10 of clinical doses or no treatment, respectively. Transconjugants were observed in all three groups, however transfer and establishment of pLFE1 were considerably enhanced in rats administered erythromycin, irrespective of dose. Practically all *E. faecalis* recipients received pLFE1 within one day in the erythromycin-treated rats, whereas four-to-five days were needed to reach a stable transconjugant population at significantly lower numbers in the non-treated rats. In addition to selection by growth, the strong favouritism of pLFE1 provided by erythromycin could possibly be explained with an increased transfer frequency. Yet, selective pressure with erythromycin was not a prerequisite for maintenance of pLFE1, because transconjugants persisted in the gut at relatively stable numbers at least 12 days after termination of antibiotic administration.

The GI tract was suggested to constitute a more favourable environment for conjugative plasmid transfer than *in vitro*, since in spite of low filter-mating frequency (~5.7×10^{-8} transconjugants/recipient) relatively high numbers of transconjugants (10^{-4} transconjugants/recipient) were observed *in vivo*, even in the non-treated rats. In order to investigate transfer of pLFE1 in a more complex model comprising an impaired colonization barrier, a similar experiment was set-up using streptomycin-treated mice. No transfer to the *E. faecalis* recipient was detected in this experiment, stressing the ability of the indigenous microbiota to reduce dissemination.

In **Paper III**, the 4031 bp nucleotide sequence of the small plasmid pLFE1 was determined and analysed. The sequence revealed an *erm*(B) gene coding for an erythromycin ribosome methylase conferring erythromycin resistance and the presence of a small erythromycin leader peptide suggested that expression of resistance may be inducible.

Plasmid pLFE1 was suggested to belong to the pMV158 family of rolling-circle-replicating (RCR) plasmids, containing a putative replication initiation protein RepB characteristic of this family. Two putative elements (a transcriptional repressor protein CopG and a small countertranscribed RNA, ctRNA), typically involved in control of RepB and thereby copy number within the pMV158 family, were also detected. A potential replication initiation site was pointed out including a double-strand origin displaying compatibility with RepB and a single-strand origin. Moreover, the presence of a secondary replication initiation site was suggested.

A sequence coding for a putative truncated mobilization protein with similarity to the pMV158 family of Pre/Mob proteins was detected. However this protein was regarded non-functional and mobilization of pLFE1 was therefore believed to require the presence of another Mob protein in *trans*.

The nucleotide sequence analysis was accompanied by practical filter-mating studies in order to determine the ability of pLFE1 to be transferred to and replicate in different species of Gram-positive bacteria. pLFE1 was shown to have a broad host-range, obtaining transconjugants from mating between donor *L. plantarum* M345 and recipients of *Lactobacillus rhamnosus, Lactococcus lactis, Listeria innocua*, *Enterococcus faecalis* and *Listeria monocytogenes*.

Resumé (Danish abstract)

Dansk titel: Overførsel af oprindelige plasmider indeholdende tetracyklin eller erythromycin resistensgener fra naturligt forekommende *Lactobacillus plantarum* stammer til andre bakterier i et tarmmiljø

Denne rapport omhandler overførsel af plasmider vha. konjugation fra naturlige laktobaciller til andre bakterier af relevans for den humane tarm. Plasmiderne indeholder gener, der koder for resistens overfor tetracyklin eller erythromycin. Konjugativ overførsel og faktorer som påvirker overførslen er blevet undersøgt *in vitro*, samt i forskellige dyremodeller, der simulerer den humane mave-tarm kanal. Det eksperimentelle arbejde omfatter aspekter, som forsøger at kaste lys over omfanget af laktobacillers medvirken til udbredelse af antibiotikaresistensgener. Dette inkluderer både overførselsfrekvenser der giver et skøn af den kvantitative udbredelse, samt ruter for overførsel, herunder hvilke bakterier der optræder som mulige recipienter. En vurdering af antibiotikas selektive virkning, både i koncentrationer under det hæmmende niveau og i koncentrationer der simulerer kliniske niveauer, er også medtaget. Derudover, er der antaget et mere mekanistisk synspunkt med fokus på de genetiske faktorer for plasmider, der bl.a. er ansvarlige for overførsel, bestemmelse af værtsspektrum, ekspression af antibiotikaresistens og regulering af replikation.

Denne rapport består dels af et teoretisk sammendrag baseret på tilgængelig litteratur indenfor området og dels af tre manuskripter, som redegør for vigtige udvalgte resultater opnået gennem projektet.
Hovedområderne i den teoretiske del er brug og virkemåder af antibiotika samt udvikling af antibiotikaresistens, inklusive ruter for udbredelse og resistensmekanismer. Derudover findes et afsnit omhandlende genoverførsel hos bakterier, hvor der bliver set mere i detaljer på den basale genetik for mobile DNA elementer samt interaktioner med deres bakterielle værter. Endeligt diskuteres overførsel af antibiotikaresistensgener i det humane mave-tarm system samt specifikke modeller, der kan anvendes til at studere dette. I den teoretiske oversigt bliver der sat ekstra fokus på tetracyklin og erythromycin samt på laktobaciller, konjugation og rolling-circle replikerende plasmider, idet de praktiske studier specielt har koncentreret sig om dette.

Manuskript I, havde til formål at teste to naturligt forekommende *Lactobacillus plantarum* stammers (DG 507 og DG 522, isoleret fra belgiske fermenterede pølser) evne til at fungere som donorer af oprindelige plasmider indeholdende erythromycin og tetracyklin resistensgener i et mave-tarm miljø. Kimfri rotter blev inokuleret med en *Enterococcus faecalis* recipient og brugt som model til at efterligne et scenarie for højst tænkelig overførsel i den humane mave-tarm kanal. Efter kolonisering med recipienten blev hver af donorstammerne introduceret og efter to dage kunne transkonjuganter detekteres i fæcesprøverne. Dette studie dokumenterede begge *L. plantarum* stammers evne til at overføre antibiotikaresistensgener vha. konjugation efter påvirkning af fysiske faktorer, svarende til dem der findes i den humane mave-tarm kanal.

I **Manuskript II**, blev overførsel af det naturligt forekommende plasmid pLFE1, indeholdende et erythromycin resistensgen, studeret i en di-associeret rottemodel, som beskrevet for manuskript I. Først blev rotterne koloniseret med en *E. faecalis* recipient og derefter blev donorstammen *L. plantarum* M345 (isoleret fra en fransk råmælksost og indeholdende pLFE1) introduceret. Udbredelsen af pLFE1 blev undersøgt i tre grupper af rotter, der henholdsvis blev behandlet med erythromycin i koncentrationer der simulerer klinisk behandling, 1/10 af klinisk behandling eller ingen behandling. Transkonjuganter blev observeret i alle tre grupper, men overførslen og etableringen af pLFE1 var tydeligt forbedret i de grupper, der havde modtaget erythromycin – uanset dosis størrelse. Stort set alle *E. faecalis* recipienter modtog pLFE1 indenfor den første dag i erythromycin-behandlede rotter, hvorimod det varede 4-5 dage at nå en stabil population og i et signifikant lavere antal for de ubehandlede rotter. Den stærke selektion af pLFE1 forårsaget af erythromycin kan foruden vækstfordelen muligvis forklares med en øget overførselsfrekvens. Selektivt pres med erythromycin var dog ikke en forudsætning for opretholdelse af pLFE1, da antallet af transkonjuganter i tarmen forblev relativt stabilt selv 12 dage efter endt antibiotikabehandling.

Mave-tarm kanalen udgjorde tilsyneladende et mere favorabelt miljø for konjugativ overførsel af plasmider end *in vitro*, idet et relativt højt antal transkonjuganter (10^{-4} transkonjuganter/recipient) kunne observeres *in vivo*, selv i ubehandlede rotter, til trods for en lav overførselsfrekvens *in vitro* (~5.7×10^{-8} transkonjuganter/recipient). For at undersøge overførsel af pLFE1 i en mere kompleks model med en svækket koloniseringsmodstand, blev et tilsvarende forsøg udført med streptomycin-behandlede mus. I dette forsøg kunne der ikke detekteres nogen overførsel til *E. faecalis* recipienten, hvilket understreger den naturlige mikrobiotas evne til at reducere overførsel.

I **Manuskript III**, blev den 4031 bp lange nukleotidsekvens for det lille pLFE1 plasmid bestemt og analyseret. Sekvensen rummede et *erm*(B) gen kodende for en erythromycin ribosom methylase, der giver resistens overfor erythromycin, og et lille erythromycin leader peptid, hvis tilstedeværelse antydede at ekspression af resistensen kan være inducerbar.

Plasmidet pLFE1 tilhører angiveligt pMV158 familien af rolling-circle replikerende (RCR) plasmider, indeholdende et potentielt replikationsprotein, RepB, som er karakteristisk for denne familie. To elementer (et protein CopG, som undertrykker transskription og et lille RNA (ctRNA) transskriberet i modsat retning), der typisk er involveret i kontrol af RepB og dermed antal plasmid-kopier i pMV158 familien, kunne også detekteres. Et muligt område for initiering af replikation, indeholdende et origin for dobbeltstrenget DNA, der er kompatibelt med RepB og et origin for enkeltstrenget DNA, blev identificeret. Derudover blev tilstedeværelsen af et sekundært område for initiering af replikation foreslået.

En sekvens kodende for et potentielt trunkeret mobiliseringsprotein i lighed med Pre/Mob proteiner fra pMV158 familien blev også fundet. Dette protein blev dog ikke regnet for funktionsdygtigt, og derfor formodedes det, at mobilisering af pLFE1 kræver et andet Mob protein i *trans*.

Analysen af nukleotidsekvensen blev ledsaget af filter-mating studier, for at undersøge hvorvidt pLFE1 kan overføres til og replikere i andre arter af Gram-positive bakterier. Det blev vist at pLFE1 har et bredt værtsspektrum, idet der blev observeret transkonjuganter fra mating mellem donorstammen *L. plantarum* M345 og recipienter af *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Listeria innocua*, *E. faecalis* og *Listeria monocytogenes.*

1. General Introduction

1.1 Background

An alarming problem exists on antibiotic resistance and increasing inability to cure infections of resistant pathogenic bacteria. In recent years focus has been directed towards commensal bacteria including lactic acid bacteria such as lactobacilli, and their contribution to dissemination and persistence of this problem. Immense support has accumulated for the argument that commensal bacteria act as reservoirs for antibiotic resistance genes found in pathogens. This amongst others owes to the fact that similar resistance determinants can be found across the "border" of pathogenicity, connecting resistance in e.g. lactobacilli with pathogenic streptococci. However, the pathways for these genetic linkages and the extent of antibiotic resistance transfer mediated by genera such as *Lactobacillus* are poorly understood.

The main pathway for exposure to lactobacilli is via ingestion. Lactobacilli are very important players in industrial food and feed production. They are often associated with a health benefit for human and animals, and many species within this group have a generally recognized as safe (GRAS) status. Yet, subsequent to ingestion lactobacilli may colonize the gastrointestinal (GI) tract becoming a part of the indigenous microbiota and/or interact with other members of the microbiota transferring antibiotic resistance genes.

Lactobacilli present in food can be either deliberately amended or occur as a result of contamination during processing. When a strain is amended to a certain product, it can be present in high numbers and its distribution follows that of the product. Potential antibiotic resistance problems associated with such strains may therefore have widespread implications. Industrial producers generally are very careful to avoid transferable antibiotic resistance genes in their starter and probiotic strains. To date, strains exhibiting resistance may be discarded, since ultimate proof of immobility of the resistance determinant can be difficult to provide. Still, by improving ability to estimate the risk associated with antibiotic resistance in *Lactobacillus* spp., companies may have an easier task developing and assessing new production strains. Furthermore, improving identification of strains posing actual risks may lead to a more firm legislation ensuring rejection of these.

Lactobacilli are common indigenous members of the gastrointestinal microbiota of various animals as well as humans. An important source of contamination with lactobacilli in food of animal origin is therefore through faecal contamination of raw-products. If the food is produced without pasteurisation or heat-treatment, which is the case for e.g. fermented sausages and ham and cheese or dairy products produced from raw-milk, survival and thus carry-over of contaminating bacteria to

the final ready-to-eat products may occur. Most European producers have very hygienic processing equipments and make a strong effort to avoid contamination of food-products. Nevertheless, complete avoidance of carry-over is not feasible. Basal knowledge of the risk associated with antibiotic resistant lactobacilli in food is therefore required.

Precautionary measures that have been taken in order to limit the flux of antibiotic resistant bacteria from animals to humans are for instance restrictions in the use of antibiotics as animal growth promoters. Yet, there is still a large overuse of antibiotics in many areas and it is well known that selective pressure induced by antibiotics leads to development and spread of antibiotic resistance. Understanding of the magnitude of antibiotic resistance dissemination mediated by lactobacilli and the effect of antibiotic pressure is however inadequate and more research is needed in this area.

1.2 Objectives of this work

The main aim of this Ph.D.-project was to study the potential for conjugation of plasmids harbouring antibiotic resistance genes from *Lactobacillus* to other bacteria in the human GI tract. Two aspects of this potential were considered namely the qualitative and the quantitative extent of transfer.

The qualitative assessment included examination of the ability of *Lactobacillus* strains to transfer resistance determinants *per se* and investigation of the pathways for spread of the plasmids. In this respect, the spectrum of different bacteria that may act as recipients and the potential of these to function as secondary distributors were analysed. Amongst potential recipients, the primary focus was held on Gram-positive species of relevance to the human GI tract, including commensals as well as opportunistic pathogens and pathogens.

Estimation of the quantitative extent of transfer in the human GI tract was another objective of this work. For this purpose, conjugation studies were set up in more or less complex environments in order to approach conditions in the human GI tract. Furthermore, it was intended to learn more about the effect of sub-inhibitory concentrations or clinical treatment with antibiotics on the transfer frequency and persistence of antibiotic resistance in the GI tract.

1.3 Research approach

Pilot experiments were performed on a number of antibiotic resistant *Lactobacillus* spp. in order to identify strains with traceable transfer capacity. These strains were used as donors in *in vitro* filter-mating experiments using primarily *Enterococcus faecalis* as recipient. *E. faecalis* is a common

member of the indigenous human microbiota, an opportunistic pathogen and known to be an efficient recipient of genetic mobile elements. Positive verification of transfer could be obtained for a limited number of donors including three wild-type strains of *Lactobacillus plantarum*, which were chosen as model organisms for further examination. These "models" had been isolated from fermented sausage and raw-milk cheese and harboured transferable plasmids conferring resistance to tetracycline, erythromycin or both.

In order to investigate dissemination of resistance plasmids after ingestion, *in vivo* experiments were set up using germ-free rats to simulate physical conditions encountered in the human GI tract. The rats were di-associated with an *E. faecalis* recipient and either of the donor models, as to allow maximal population densities of test bacteria and thus constitute worst-case scenarios representing upper limits of transfer. The rats were divided into groups receiving no antibiotics (controls), sub-inhibitory or clinical doses. Results were compared with those from experiments conducted on streptomycin-treated mice applying similar protocols, in order to assess the effect of a colonization barrier.

To facilitate a better understanding of some of the factors determining e.g. mobilization and the quantitative dissemination as well as the host-range of plasmid transfer observed for model strain *L. plantarum* M345 *in vivo* and *in vitro*, sequencing and analysis of the respective plasmid pLFE1 was performed.

2. Antibiotics

2.1 Problems with antibiotic resistance

The major risk of antibiotic resistance is obviously failure of treatment of a specific infection. Antibiotic resistant infections can result in increased morbidity due to a delay in the curing-process if the first choice of antibiotic fails or due to accompanying diseases caused by the antibiotic treatment. In some instances, treatment in due time is mandatory, and an unsuccessful attempt of curing will be mortal. In other cases, alternative treatments may be absent or insufficient for instance due to multi-resistant infections. An earlier study estimated that the duration of hospital stays, the morbidity and the mortality were doubled compared with antibiotic-susceptible infections (Holmberg et al. 1987). However, the extent and costs of antibiotic resistance is very difficult to assess and probably vary considerably depending on e.g. the knowledge of resistance patterns of the infectious agent and the current antibiotics available. In all circumstances, antibiotic resistance has great economic and societal implications and poses a radical threat to human and animal health.

It is well established that the extent of antibiotic usage is tightly linked to development of antibiotic resistance and introduction of new compounds on the market is by convention followed by emergence of bacterial resistance (Levy and Marshall 2004). Exposing bacteria to antibiotics results in a selective advantage to those, which have developed or acquired resistance towards it, and the resistant bacteria can therefore spread at the expense of the sensitive ones (Moubareck et al. 2003). The nature of selection should result in practical action in compliance with guidelines for the prudent use of the antibiotics, in order to retain their activity. For clinical use, this means a proper diagnosis, careful choice of antibiotic agent that preferably should have a narrow range of activity and time- and dosis-controlled accomplishment of treatment. In modern food animal production antibiotics are also used in large amounts and dissemination of antibiotic resistant bacteria from these animals to humans is possible via the food chain. The antibiotics can be used in any of four different ways, (i) therapy of clinically sick animals, (ii) metaphylactics, which is treatment of healthy animals present in a pen including sick animals, (iii) prophylactics, which is preventive treatment of animals exposed to stressed situations or (iv) growth promotion, which is continuous application of antibiotics at subtherapeutic concentrations to feeds in order to improve animal growth (Aarestrup 2005).

Especially the application of antibiotics as growth promoters in animal husbandry has received a lot of criticism. The sole objective of growth promotion is an economic gain by obtaining a faster meat production line. The way to achieve this is by long-term feeding with antibiotics at low

concentrations, which is associated with an extended risk of resistance development. Despite difficulties in providing direct evidence that the ultimate outcome of this procedure is an increase in antibiotic resistant human pathogens, the precautionary principle led to a more strict legislation in EU. Hence, in 1970, the European Commission banned tetracycline as an agent for growth promotion. Sweden thereafter acted as a pioneer nation taking up a more progressive attitude against antibiotics for growth promotion by proclaiming a total ban in 1986. Detection of vancomycin resistant enterococci in food animals led the Danish food industry to decide on a voluntary abolishment of the growth promoter avoparcin in 1995, in order to prevent cross-resistance to the important human drug vancomycin (Aarestrup 2000). In the late 1990s, the European Commission extended their ban to include drugs relevant for human therapy or capable of causing cross-resistance to therapeutically relevant agents (Chopra and Roberts 2001) and from January 2006 a total abolishment of antibiotics used for animal growth promotion was decided (European Commission 2005). In most countries outside Europe however, restrictions on this area are lacking behind.

A large number of studies and also nationwide monitoring programs have investigated the effect on antibiotic resistance after cessation of antibiotic use (Aarestrup et al. 2001;Boerlin et al. 2001;Klare et al. 1999;Seppala et al. 1997;Van Den Bogaard et al. 2000). Generally, these studies report a decrease in the occurrence of resistance after removal of the selective pressure. However, some studies have shown status quo conditions, which in many cases have been assigned to co-selection of the antibiotic resistance determinant either by coupling of the determinant with other antibiotic resistance markers, heavy-metal resistances etc. Since the metabolic burden of maintaining the resistance determinants sometimes is relatively low, the loss of resistance can also be very slow, and even after a long time resistant isolates may persist in the environment. This resistant subpopulation can after recurring selection very quickly grow to reach initial numbers (Levy and Marshall 2004).

It has been suggested that the fastest way to eliminate resistant isolates is to out-compete and replace them with susceptible ones (Levy and Marshall 2004). The success of this strategy depends on the availability of susceptible strains in the adjacent environment. Thus, a chicken, which belongs to a flock with high resistance preference or a patient on a hospital surrounded by other patients in antibiotic treatment, may continuously be reinfected with resistant isolates. Therefore, a global effort to limit the use of antibiotics is necessary to control antibiotic resistance dissemination.

2.2 Routes of antibiotic resistance dissemination

Bacteria are a ubiquitous part of our surroundings and there is a continuous movement and exchange either faster or slower in the bacterial populations inhabiting different environments. The main focus of this Ph.D project has been on dissemination of antibiotic resistance within the food chain with special emphasis on transfer from lactic acid bacteria present in ingested food to bacteria inhabiting the human GI tract. The following paragraph will therefore be limited to discuss genetic exchange related to this area.

During the last decade, increasing attention has been paid to the relevance of commensal bacteria acting as a pool of antibiotic resistance genes for human pathogens (Levy and Marshall 2004;Mathur and Singh 2005;Salyers and Shoemaker 1996;Teuber et al. 1999;Teuber and Perreten 2000). The main pathway for human uptake of bacteria and transportation to the GI tract is via the food chain. Food products contain bacteria that can be either deliberately amended to initiate food ripening, add flavour etc, or which are present due to contamination of the products during processing. In the case of deliberate addition of bacteria, knowledge of antibiotic resistance is of course much easier to achieve and generally strictly controlled by the modern food producers. However, in some, mainly smaller production facilities of e.g. cheese, the food processing occurs after old traditions involving back-sloping of a non-defined bacterial culture. These facilities commonly have a long history of safe use, but the non-defined bacterial starter culture may be associated with a higher risk if it consists of commensals carrying antibiotic resistance determinants (Maietti et al. 2007).

In food based on animal products such as meat and milk, carry-over of bacteria from animal skin and faeces to the raw-products is inevitable during milking and slaughtering. If the contaminated raw-products are not sufficiently heat-treated by pasteurization of the milk or cooking of the meat, the bacteria will be directly transported to the human intestine after ingestion, where they may colonize and/or interact with the resident microbiota by disseminating resistance genes. Raw-milk cheeses and fermented sausages are examples of products that generally are associated with higher numbers of animal transmitted bacteria, since the relatively gentle manufacturing procedure allows for survival or even multiplication of these into the final ready-to-eat product (Teuber and Perreten 2000). This exchange of bacteria from animals via the food to the consumers has been suggested to contribute significantly to human antibiotic resistant infections, especially when enteric organisms are involved (Levy and Marshall 2004).

2.3 Use of antibiotics

2.3.1 Macrolides

Macrolides are a group of natural or semisynthetic antibiotics composed of a lactone ring of variable size to which one or more sugars and/or amino groups are attached via glycosidic bonds (Table 1)(Mazzei et al. 1993). The prototype of macrolides is erythromycin, which is produced by *Saccharopolyspora erythraea* and was the first compound of the group to be introduced in 1952 (Roberts et al. 1999). In the 1980s, semisynthetic derivatives of erythromycin were developed including the clinically important roxithromycin, clarithromycin and azithromycin, which essentially shared the antibiotic mechanism of action but had improved pharmacokinetic abilities, less adverse effects and a broader spectrum of activity (Roberts 2004). Other compounds belonging to the class are the 16-membered macrolides, which are only available in certain countries, e.g. spiramycin and tylosin used for animal growth promotion (Leclercq 2002).

Table 1: Common natural and semisynthetic macrolide antibiotics

lactone ring	natural	semisynthetic
14-membered	erythromycin	dirithromycin roxithromycin clarithromycin
15-membered		azithromycin
16-membered	josamycin midecamycin spiramycin	rokitamycin miocamycin tylosin

Revised from (Mazzei et al. 1993) with changes adapted from (Leclercq 2002).

The macrolides are active against Gram-positive bacilli and cocci including *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus* as well as Gram-negative diplococci such as *Neisseria gonorrhoeae.* Furthermore, they inhibit *Chlamydia trachomatis* and some species of *Mycoplasma* and *Chlamydophila* (Lægemiddelkataloget 2008). Gram-negative bacilli are generally resistant to macrolides because the outer membrane offers protection from penetration by these hydrophobic compounds (Russell and Chopra 1990). However, some important exceptions are e.g. *Legionella* spp., *Campylobacter jejuni* and *Helicobacter pylori* (Leclercq 2002). Some of the semisynthetic derivatives show a broader range of activity than that of erythromycin, particularly clarithromycin with a greater potency against anaerobic bacteria and azithromycin with the inclusion of e.g. some Gram-negative enteric bacilli and *Haemophilus* spp. (Leclercq and Courvalin 1991).

Macrolides are primarily used to treat patients that are intolerant to penicillins and are suffering from e.g. pneumoni, tonsillitis, otitis, sinusitis and wound infections caused by *Streptococcus pneumoniae* or β-hemolytic streptococci. Furthermore the macrolides and especially azithromycin are used to threat infections of the urinary tract and cervix caused by *Chlamydia* and has also been effective in the treatment of gonorrhoea, however, the application for the latter purpose is declining due to increasing resistance (Lægemiddelkataloget 2008).

In Denmark, the overall consumption of macrolides for human clinical treatment decreased by 4.2% in 2006 compared to 2005. This decrease was primarily due to a decrease in the use of erythromycin, which has been slightly but constantly decreasing since 1998. Furthermore, the development in the last years increasing consumption of roxithromycin was arrested from 2005 to 2006. Despite the decreasing amount of consumed macrolides, this group of antibiotics still remains to be the third most used in the Danish primary health care sector with an annual consumption in 2006 corresponding to 2.31 defined daily doses (DDD) per 1000 inhabitant-days. In the hospital sector the consumption of macrolides for nosocomial infections also decreased to represent 4.5% of the total consumption in 2006 (DANMAP 2006).

The way of antibiotic action is similar in all macrolides, however there is small variations in their antibiotic spectrum and considerable differences in their pharmacokinetics. The clinical use of erythromycin is associated with much more adverse effects than that of the other macrolides clarithromycin, roxithromycin and azithromycin (Lægemiddelkataloget 2008). In the new Danish guidelines from 2006, clarithromycin is recommended as the first-choice of macrolides (DANMAP 2006).

2.3.2 Tetracyclines

The first tetracyclines to be discovered were chlortetracycline and oxytetracycline, which were derived as naturally occurring products from different species of the soil bacterium *Streptomyces* in 1948. Several other derivatives including semisynthetic molecules have been marketed over the years in order to improve and increase the applicative abilities of the tetracycline group and to circumvent problems with resistance to the older analogs (Table 2). After a long period with increasing resistance to the existing tetracycline compounds and no new products on the market, the US food and Drug Administration (FDA) approved tigecycline for human clinical trials in 2005. Tigecycline is the first compound belonging to a new generation of tetracycline derivatives named glycylcyclines (Doan et al. 2006).

Table 2: Principal members of the tetracycline class.

Generation	Generic name	Origin	Year of discovery	Status
I	Chlortetracycline	*S. aureofaciens*	1948	marketed
	Oxytetracycline	*S. rimosus*	1948	marketed
	Tetracycline	*S. aureofaciens, S. rimosus, S. viridofaciens*	1953	marketed
	Demethylchlortetracycline	*S. aureofaciens*	1957	marketed
	Rolitetracycline	semisynthetic	1958	marketed
	Limecycline	semisynthetic	1961	marketed
II	Methacycline	semisynthetic	1965	marketed
	Doxycycline	semisynthetic	1967	marketed
	Minocycline	semisynthetic	1972	marketed
III	Tigecycline	semisynthetic	1993	marketed

Adapted from (Chopra and Roberts 2001) with minor updates.

Tetracyclines are broad-spectrum agents with activity against both Gram-positive and Gram-negative bacteria as well as chlamydiae, mycoplasmas, Rickettsiae, protozoan parasites etc. Due to their wide range of activity, low cost and absence of noteworthy side effects, the tetracyclines have been extensively used for treatment of many different human and animal infections, but their applications also includes prophylactic use against malaria, control of infections in aquaculture, fruit production, growth promotion in animal husbandry and many other (Chopra and Roberts 2001). The first tetracyclines were approved by FDA for animal growth promotion in 1951 and the amount of drugs added at subtherapeutic levels to animal feeds since then has been excessive (Chopra and Roberts 2001). In Europe tetracyclines were banned as growth promoters in the early 1970s, however tetracyclines are still used for animal growth promotion in many countries outside Europe.

Tetracyclines can be used in the treatment of various infections of the respiratory tract, urinary tract, bowel and many other local and systemic diseases. However, the applicability of these drugs has experienced a severe reduction due to increasing resistance in the causative organisms. Still, tetracyclines are important agents especially against community-acquired infections. In Denmark they belong to the fourth most used class of antibiotics in the primary health care section representing 1.38 DDD per 1000 inhabitant-days in 2006. Tetracycline and doxycycline composed the majority of consumed tetracyclines and was primarily used for the treatment of acne and for malaria prophylaxis, respectively (DANMAP 2006).

2.4 Mechanisms of antibiotics and countermoves of resistance

2.4.1 Macrolides

Macrolides are protein-inhibiting antibiotics, which function by binding to the bacterial 50S ribosomal subunit in a reversible manner, thereby inhibiting peptide elongation and thus exercising a bacteriostatic effect. Although the macrolides are structurally unrelated to the lincosamides and streptogramin B antibiotics, their binding sites within the bacterial ribosome are overlapping. Therefore, dependent on the resistance mechanism, the host bacteria show resistance either to one, two or all three groups of antibiotics. The mechanisms of acquired resistance genes towards these antibiotics include rRNA methylases, which function by ribosomal protection, efflux proteins and several different inactivating enzymes (Table 3). The former mechanism confers resistance towards all three groups, whereas the latter two normally confer resistance to only one or two of the groups (Roberts 2004).

Table 3: Mechanism of resistance for characterized MLS resistance genes. Revised from (Roberts 2008).

Ribosomal protection, n = 32	Efflux, n = 14	Enzymatic inactivation, n = 6	
rRNA methylases[a]	ATP-transporters or major facilitators[b]	esterases[c]	phosphorylases[d]
erm(A), *erm*(B), *erm*(C), *erm*(D), *erm*(E), *erm*(F), *erm*(G), *erm*(H), *erm*(N), *erm*(O), *erm*(Q), *erm*(R), *erm*(S), *erm*(T), *erm*(U), *erm*(V), *erm*(W), *erm*(X), *erm*(Y), *erm*(Z), *erm*(30), *erm*(31), *erm*(33), *erm*(34), *erm*(35), *erm*(36), *erm*(37), *erm*(38), *erm*(39), *erm*(40)	*msr*(A), *msr*(C), *msr*(D), *car*(A), *ole*(B), *ole*(C), *srm*(B), *tlc*(C), *lsa*(A), *lsa*(B), *vga*(A), *vga*(B), *mef*(A), *lmr*(A)	*ere*(A), *ere*(B)	*mph*(A), *mph*(B), *mph*(C), *mph*(D)

[a] resistance to MLS_B; [b] resistance to either lincomycin, oleandomycin, spiramycin, tylosin or streptogramin A, or both erythromycin and streptogramin B; [c] resistance to erythromycin; [d] resistance to macrolides (Roberts 2004).

The most frequently occurring resistance mechanism towards the macrolide, lincosamide and streptogramin B (MLS_B) antibiotics is facilitated by the rRNA methylases. These enzymes mediate a posttranscriptional methylation of a specific adenine residue in the 23S rRNA, which subsequently inhibits binding of MLS_B to the 50S ribosomal subunit. Thereby the obstructing effect of the antibiotic is prevented. rRNA methylases can be either constitutive or inducible, but not all of the MLS_B function equally well as inducers. Though, generally erythromycin is efficient to turn on the inducible genes, whereas lincomycin and streptogramin B often are not (an exception is in some streptococcal isolates). The inducible characteristic implies that the host will appear susceptible when exposed to a non-inducing MLS_B antibiotic in an un-induced stated, but will assume resistance in an induced state (Roberts et al. 1999).

The majority of rRNA methylase genes was initially described in Gram-positive bacteria and also has a low G+C content suggestive of a Gram-positive origin or was discovered in *Streptomyces* spp. Typically, these genes are associated with mobile elements such as transposons or plasmids, which facilitate their dissemination. However, the host-range of the genes seems highly variable with a large proportion only being isolated from a single genera (for instance 1/3 of the genes are found exclusively in *Streptomyces* spp.) and another part of the genes have been discovered in a large number of different genera (Roberts 2004). However, the number of genera in which the individual genes are found seems to be increasing. Currently, the rRNA methylase gene with the widest distribution is *erm*(B), which have been found in 33 genera of both Gram-positive and Gram-negative, aerobic and anaerobic bacteria inhabiting many different environments. A common coupling of *erm*(B) is with conjugative Tn*916*-like transposons in linkage with the tetracycline determinant *tet*(M) (Roberts 2004).

The efflux genes involve ATP transporters and major facilitator transporters, which all function by lowering the intracellular concentration of the antibiotic via active pumping out of the cell or the cellular membrane. The majority of these genes, i.e. 11 out of 14 genes, are currently isolated solely from single genera (*Streptomyces*, *Staphylococcus, Streptococcus* or *Enterococcus*). In contrast, the most widespread of the efflux genes *mef*(A), which encodes resistance to erythromycin has a very broad host range and is found in 24 different genera (Roberts 2008). However, whether the difference in host spectrum relies on the association of the individual resistance genes with mobile elements of various range or other factors is to this point uncertain (Roberts 2004).

Amongst the inactivating enzymes that confer resistance to macrolide antibiotics by modification of the drug, two esterases (Ere) and four phosphorylases (Mph) have been described. These genes have so far been detected in a moderately broad selection of predominantly Gram-negative bacteria. Though, the likelihood of identifying the genes in a more widespread number of genera is plausible, since relatively few studies have been attended to this area (Roberts 2004).

2.4.2 Tetracyclines

Tetracyclines inhibit protein synthesis by binding to the ribosomal 30S subunit. The binding of the antibiotic to the ribosome is reversible and so its bacteriostatic effect. In enteric Gram-negative bacteria, the tetracyclines cross the outer membrane into the periplasm via the OmpR and OmpC porin channels as positively charged cation-complexes. In the periplasm, the complex is believed to dissociate and the lipophilic tetracycline molecule can thereafter diffuse into the cytoplasm through

the lipid bilayer of the cytoplasmic membrane. In Gram-positive bacteria, the form of the tetracycline molecule that traverses the cytoplasmic membrane is also assumed to be the electroneutral lipophilic form. Uptake through the cytoplasmic membrane requires energy and is mediated by the pH gradient of the proton motive force. The environment within the cytoplasm is likely to enforce chelation of the tetracycline molecule and the active species that binds to the bacterial ribosome is assumed to be a Mg^{2+}-tetracycline complex (Chopra and Roberts 2001). Upon interaction between the ribosome and the tetracycline complex a change in the secondary structure of the ribosome is induced. This structural rearrangement prevents the association of aminoacyl-tRNA to the bacterial ribosome thus inhibiting elongation of the growing polypeptide by addition of new amino acids (Connell et al. 2003).

Studies of bacterial populations existing prior to marketing of tetracyclines in the early 1950s have documented that the development of resistance towards this class of antibiotics is a phenomenon that have emerged in concert with introduction of the drugs (Hughes and Datta 1983). Nowadays, resistance is widespread in both Gram-positive and Gram-negative commensal and pathogenic bacteria first and forwards due to dissemination of tetracycline (*tet*) and oxytetracycline (*otr*) resistance determinants providing efflux- and ribosome-based resistance. Currently, 38 different acquired *tet* genes have been described of which the majority are energy-dependent efflux pumps, some are ribosomal protection proteins and a few code for inactivating enzymes or functions by a so far unknown mechanism (Table 4). The classification of genes conferring resistance to tetracyclines have been based on sequence homology, with genes showing more than 80% amino acid identity being given the same gene designation.

Table 4: Mechanism of resistance for characterized *tet* and *otr* resistance genes. Adapted from (Roberts 2005).

Efflux n = 23	Ribosomal protection n = 11	Enzymatic inactivation n = 3	Unknown n = 1
tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(V), *tet*(Y), *tet*(Z), *tet*(30), *tet*(31), *tet*(K), *tet*(L), *tetA*(P)[a], *otr*(B), *tcr*(3), *tet*(33), *tet*(35)[b], *tet*(38), *tet*(39), *otr*(C)	*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q), *tet*(T), *otr*(A), *tetB*(P)[a], *tet*, *tet*(32), *tet*(36)	*tet*(X), *tet*(34), *tet*(37)	*tet*(U)

[a] *tetA*(P) and *tetB*(P) are counted as one operon and *tetB*(P) is not found separately.
[b] not related to other *tet* efflux genes

The efflux genes code for energy-dependent membrane-associated proteins, which reduces the concentration of tetracycline within the bacterial cell by an active efflux pumping mechanism. Hence, protons are transferred against a gradient into the cell in exchange for tetracycline-cation

complexes. Of the 23 known efflux genes, three have been derived from the originally antibiotic-producing genera *Streptomyces*, four genes are from Gram-positive bacteria and the latter 16 genes originate from Gram-negative bacteria. The efflux genes are found both in Gram-positive and Gram-negative bacteria and are the most prevalent found *tet* genes in Gram-negative bacteria (Roberts 2005).

The preserving functions of the bacterial ribosomal protection proteins (RPPs) have not been entirely elucidated. The Tet(O) and Tet(M) are the most thoroughly studied RPPs belonging to this class but the other members are assumed to share similar mechanisms. A functional model has been proposed, where the RPPs hinder ribosomal binding of the antibiotics by interacting with the primary antibiotic binding site within the ribosome thus causing an allosteric disruption of the site. Hereby, the dissociation constant between the tetracyclines and the bacterial ribosome has been shown to increase significantly. However, whether the RPPs actively prevent rebinding of the tetracyclines after their release is unclear (Connell et al. 2003).

It has been proposed that bacteriostatic antibiotics such as tetracycline expose a higher risk for development of antibiotic resistant populations. By treatment with bacteriostatic agents, the sensitive microbial population will be arrested in growth but not killed immediately. A large potential recipient population may therefore be available and transconjugants emerging will be favoured by the selective pressure (Licht et al. 2003). Furthermore, a specific property reported for tetracycline is the inducing effect on transfer frequency of the conjugative transposon family Tn*916*-Tn*1545* conferring resistance to tetracycline (Showsh and Andrews, Jr. 1992). So far, this effect has not been described for other antibiotic resistance elements. However, in terms of risk assessment, this is a highly concerning property, since treatment with the antibiotic not only leads to selection by vertical transfer but also increases the horizontal dissemination.

2.5 Resistance – intrinsic and acquired

Intrinsic resistance (also called natural resistance) is an inherent trait that often characterizes all strains belonging to a given species. Oppositely, a strain that is member of a typically susceptible species is regarded to have acquired the resistance trait if it deviates from the normal pattern by showing a resistant phenotype (Normark and Normark 2002). When evaluating the risk of antibiotic resistance dissemination, distinguishing between intrinsic and acquired resistance is an important parameter (Fig. 1). Although transfer of resistance from an intrinsically resistant strain to other bacteria cannot be excluded, the possibility is considered minimal. Acquired resistance is either a

result of alteration of indigenous genes by point mutations, deletions, inversions or insertions or gain of exogenous genes by horizontal gene transfer (Normark and Normark 2002). The risk of transfer of the former can be compared to that of intrinsic resistance, whereas a high risk of transfer is associated with the latter, especially if the added genes are harboured on mobile elements (Mattila-Sandholm et al. 1999;Vankerckhoven et al. 2008).

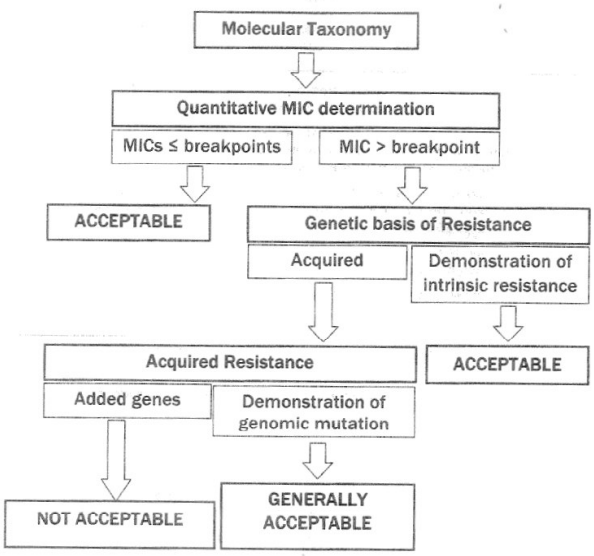

Fig. 1: Proposed scheme for the antibiotic resistance assessment of a bacterial strain used as a feed additive. Adapted from (European Commission 2005).

In practice, differentiation between intrinsic and acquired resistance is not always clear-cut. First it must be established whether the strain is resistant or susceptible. This determination is based on the minimum inhibitory concentration (MIC) of the strain and the microbiological breakpoint for the species and antibiotic agent in question. The MIC value is the lowest concentration of the antibiotic that inhibits bacterial growth and several methods for MIC determination have been reported including the sensititre system (Floréz et al. 2005) agar dilution (Butaye et al. 2000;Floréz et al. 2005), microbroth (D'Aimmo et al. 2007;Klein et al. 2000), E-test (Danielsen and Wind 2003;Hummel et al. 2007) and disc diffusion (Huys et al. 2002;Perreten et al. 1998). Disc diffusion and E-test probably are the most widespread methods and are based on similar principles; the test bacterial culture is streaked onto an agar plate and growth inhibition zones adjacent to pre-manufactured antibiotic-containing implements placed on the agar plate are measured subsequent to inoculation. E-test involves application of an antibiotic test strip with a built-in gradient and the

MIC value can be read directly from the bacterial growth zone along this gradient. In disc diffusion, a disc with a specific antibiotic concentration creates a diffusion gradient in the agar away from the disc. Measurement of the distance between the disc and the bacterial growth zone can thereafter be converted to a MIC value (Huys et al. 2002). Sometimes a haze or pinpoint colonies appearing within the inhibition zone can render the reading of MIC values difficult and subject to large variation depending on the examiner. A standardised protocol is an important prerequisite for the ability to reproduce and compare different MIC values. However, application of a single protocol for all species and antibiotic agents is not feasible, since optimization of growth conditions for the individual species and avoidance of interactions between growth media and antibiotic agent should be attended to. Until recently, protocols for testing of non-clinical isolates such as lactic acid bacteria have been in short supply. However in recent years this area have received much attention and although no official standard protocols have been made available so far, researchers have put a large effort into recommendations for formulation of such (Danielsen et al. 2004;Danielsen and Wind 2003;Huys et al. 2002;Vankerckhoven et al. 2008).

Establishment of the microbiological breakpoint for a given antibiotic and species is based on the statistical distribution of MIC values for a whole group of strains presumed to be representative for that species. Ideally for establishment, the determined MIC values depict a normal distribution with a narrow concentration range. In this situation, the microbiological breakpoint can be set at the upper concentration of MICs for this cluster (Florez et al. 2007). Sometimes all strains show high MICs for a given antibiotic and the species is thus considered to be intrinsically resistant. Though, if the number of investigated strains is limited, particular notice should be given to the possibility that all strains tested may have received acquired resistance genes. Likewise, attention should be paid to the inability of this phenotypic method to detect acquired resistance genes in a background of intrinsic resistance. On the contrary, if a microbiological breakpoint is set and outliers with MICs above this breakpoint concentration are pointed out, the strains can be considered to have acquired resistance. In the light of a risk evaluation, these strains should be subject to further analysis in order to establish the genetic basis for resistance (European Commission 2005;Florez et al. 2007;Kastner et al. 2006;Vankerckhoven et al. 2008).

2.6 Antibiotic resistance in *Lactobacillus* spp.

In recent years, several investigations have been undertaken in order to elucidate the occurrence of antibiotic resistance in lactobacilli. These investigations have been hampered by the lack of

standardised methods for MIC testing, lack of well-founded microbiological breakpoint values and large species variation in antibiotic resistance phenotypes. Therefore a process was initiated in order to create a basis for distinguishing susceptible strains from strains with intrinsic resistance or acquired and thus putative transferable resistance (Danielsen et al. 2004;Danielsen and Wind 2003;Huys et al. 2002). A good fundament has now been established, but there is still call for optimization of methods and adjustment of microbiological breakpoint values as more strains are being tested (Ammor et al. 2007;Vankerckhoven et al. 2008).

Generally, lactobacilli have been reported to be intrinsically resistant to the folic acid metabolism inhibitors trimethoprim and sulphonamides. Folic acid is used e.g. for the synthesis of purines, which is required as an external supplement for many lactobacilli to be able to grow. Lack of the folic acid synthesis pathway and thus the target for trimethoprim and sulphonamides has therefore been suggested as the cause of resistance (Katla et al. 2001). Dihydrofolate reductase (DHFR) is the specific target for trimethoprim and is responsible for the last step in the folic acid pathway converting dihydrofolate to the active tetrahydrofolate. An early study has shown that some lactobacilli contain DHFRs that are insensitive to trimethoprim and proposed this quality to be connected with resistance (Then and Riggenbach 1978). This finding is in correspondence with a recent Danish study, which tested the effect of bacterial growth medium composition on trimethoprim susceptibility (Danielsen et al. 2004). Here it was stated that lactobacilli do not have intrinsic resistance to trimethoprim. Rather, the presence of thymidine in MRS medium commonly used for MIC testing was suggested to be the cause of erroneous conclusions on intrinsic resistance. Thymidine antagonizes the action of trimethoprim but was excluded from the medium in the experiments performed by Then and Riggenbach, thus making their results more reliable.

Resistance to the cell wall inhibiting glycopeptide, vancomycin is widespread among lactobacilli. In this case lack of the vancomycin target *D*-Ala-*D*-Ala dipeptide is responsible, since most *Lactobacillus* species contain *D*-Ala-*D*-Lactate in the peptidoglycan layer instead, thus providing intrinsic resistance (Ammor et al. 2007). Other inhibitors of the cell wall synthesis such as β-lactamase inhibitors and the penicillins piperacillin and ampicillin are normally active against lactobacilli whereas higher resistances are observed toward oxacillin and cephalosporins and large variation exists in the resistance profiles of different *Lactobacillus* species to bacitracin. Resistance to cell wall synthesis inhibitors is mainly caused by impermeability of the cell wall due to the absence of cytochrome-mediated electron transport. Contributing to the different resistant patterns

between species are however also multidrug transporters and defective cell wall autolytic systems (Ammor et al. 2007).

Altogether, lactobacilli are susceptible to antibiotics inhibiting the synthesis of proteins such as tetracycline, erythromycin, clindamycin and chloramphenicol, whereas higher tolerance towards aminoglycosides such as neomycin, kanamycin, streptomycin and gentamicin largely seems to be an inherited characteristic (Ammor et al. 2007;Hummel et al. 2007).

The frequency of *Lactobacillus* strains, which deviates considerably from the normal susceptible population, is usually low. Amongst a selection of 45 LAB probiotic or starter strains (including 17 *Lactobacillus* strains) analysed for various antibiotic resistances, Hummel and coworkers reported only one non-wild-type isolate, which harboured the *erm*(B) gene conferring resistance to erythromycin (Hummel et al. 2007). Likewise, a study of 189 Norwegian LAB starter strains of which 55 were different species of *Lactobacillus*, reported only one isolate to be resistant. This isolate showed high-level streptomycin resistance, but the genetic basis for the resistance was not investigated (Katla et al. 2001). In another extensive examination of antibiotic resistance in 473 LAB strains predominated by lactobacilli and including probiotic and starter strains, 0.8% of the strains exhibited phenotypic resistance to streptomycin, erythromycin or clindamycin and 3.1% were resistant to oxytetracycline. Isolates with MICs exceeding the microbiological breakpoint values were further subjected to PCR analysis for detection of known resistance genes. No streptomycin resistance genes were detected but in some of the isolates *tet*(W), *tet*(M) or *erm*(B) were found, presumably explaining the observed resistances (Klare et al. 2007). Similarly, a study including 74 *Lactobacillus* strains from Swiss starter and probiotic cultures examined phenotypically deviating isolates for resistance genes by microarray hybridization. The presence of *tet*(W) and *lnu*(A) conferring resistance to tetracycline and lincosamide, respectively, was confirmed in one *L. reuteri* isolate and the latter determinant was found to be associated with a plasmid.

Antibiotic resistant *Lactobacillus* strains have also been isolated from a range of different food products such as fermented drinks and yoghurts (D'Aimmo et al. 2007;Temmerman et al. 2003), cheese (Catatolok and Gogebakan 2004;Florez et al. 2005), fermented sausages (Gevers et al. 2000) and raw and processed pork and chicken meat products (Aquilanti et al. 2007). Different studies have also looked at resistance in lactobacilli originating from human samples, and both relatively low incidences (Charteris et al. 1998) and extraordinary high incidences of resistances have been reported (Catatolok and Gogebakan 2004).

Many different types of antibiotic resistance determinants have been detected in lactobacilli, for instance the chloramphenicol resistance gene *cat*, the aminoglycoside resistance gene *aph* and several different genes conferring resistance to erythromycin *mef*(A) and the MLS_B antibiotics *erm*-(B, C, G, F, T) or tetracycline *tet*-(K, M, O, Q, S, W, L, Z, 36). The most frequently occurring determinants, however, seem to be *erm*(B) and *tet*(M) (Ammor et al. 2007;Aquilanti et al. 2007;Catatolok and Gogebakan 2004;Roberts 2008).

Overall, the number of studies, which have investigated transferability of native antibiotic resistance determinants from lactobacilli, is limited. A Belgian study confirmed conjugation of tetracycline and erythromycin resistance plasmids from a range of different *Lactobacillus* species to *E. faecalis* and *L. lactis* recipients by *in vitro* filter mating (Gevers et al. 2003b), but most of the performed studies have failed to detect transferability (Hummel et al. 2007;Kastner et al. 2006;Klare et al. 2007). Negative transfer results are however not unequivocal proof of immobility of these determinants and in general more analyses are required to shed light on the mobility of acquired antibiotic resistance determinants in *Lactobacillus.*

3. Gene transfer

Gene transfer is a very important means of bacterial adaptation to changing environments. Generally three major mechanisms of horizontal gene transfer (HGT) between bacteria exist: transformation, transduction and conjugation. Transformation was the first mechanism of HGT to be discovered. The process involves active uptake of DNA released from the surroundings and subsequent integration and replication in the new host. In order to accomplish the first step concerning uptake of free DNA into the cell, the bacteria need to be competent. Amongst bacteria analysed for natural competence, approximately 1-2%, including both Gram-positive and Gram-negative bacteria has been shown to possess this ability (Jonas et al. 2001;Thomas and Nielsen 2005). In as far as the free DNA is comprised of chromosomal genetic material, incorporation by homologue recombination in the new host is necessary. This step usually confines transformation to closely related species (Thomas and Nielsen 2005). During the last decade, increased marketing of products containing genetically modified organisms (GMOs) has attracted attention to the risk of transformation of genetic material in the mammalian gut. However, experiments dealing with this topic have found no indications of transformation of genetically modified plant DNA to bacteria in an intestinal environment (van den Eede et al. 2004).

Transduction is the process of HGT mediated by bacteriophages, which are bacterial viruses. Some bacteriophages can during the lytic cycle incorporate parts of the host DNA into their capsid by accident. Upon lysis of the host cell these so-called transducing and usually virally defective particles are released and can be reinjected into new host cells. The injected donor genes must hereafter be incorporated by homologous recombination into the recipient in order to be maintained – a process known as generalized transduction (Madigan et al. 1997). A second mechanism is known as specialized transduction and involves the accidental incorporation of host DNA directly into the genome of a temperate bacteriophage. In contrast to the virulent bacteriophage, which only option is to follow the lytic pathway, the temperate bacteriophage can choose a more passive pathway entering a lysogenic circle in the host. This implies another possibility than homologous recombination for maintenance of the host DNA – namely the integration of the modified bacteriophage genome into the host chromosome and simultaneous replication as a prophage (Madigan et al. 1997). In more seldom instances, the genome of the bacteriophage may also replicate independently in the host as a circular or linear plasmid (Frost et al. 2005).

Investigations of the extent of HGT by transduction in the GI tract are scarce, though some studies have reported that transduction in this environment can take place (Acheson et al. 1998;Cornick et al. 2006). The narrow host-range of most bacteriophages implies that dissemination by this mechanism mostly occurs among bacteria of close relation. However, compilation of data from recent years sequencing projects has revealed extensive mosaicism in bacteriophage genomes. This finding has led to a reassessment of the significance of transduction. Hence the widespread distribution of bacteriophages combined with their extensive numbers may increase the chance of both homologous and non-homologous successful recombination, thus providing a larger input to bacterial plasticity than previously anticipated (Hendrix 2003).

Conjugation is the third mechanism of HGT and is still regarded as the most important contributor to bacterial gene shuffling. Conjugation has potential to occur at a very high rate and between phylogenetically very distinct bacteria - even inter-kingdom transfer has been reported. In contrast to transformation and transduction, a prerequisite for conjugal transfer is intimate contact between donor and recipient bacteria. In Gram-negative bacteria, the mechanism for establishment of cell-to-cell contact is well known and shown to take place via complex extracellular filaments named sex pili. In contrast, the corresponding establishment of physical contact between Gram-positive bacteria is more elusive (Grohmann et al. 2003). The mediator of this establishment are the so-called transfer (*tra*) genes that can be harboured either on large conjugative plasmids (>20 kb),

conjugative transposons or on the chromosome. Transposons are mobile elements, which are devoid of an autonomous replication machinery and therefore depend on the integration and replication in conjunction with the chromosome or a plasmid. Transposons contribute significantly to the spread of antibiotic resistance genes however, in this Ph.D-project focus has been on conjugation mediated by plasmids, therefore the following paragraph will be subjected to this field.

3.1 Plasmids

Plasmids are extrachromosomal and usually circular elements of DNA that contain their own genes for initiation and control of replication. The plasmids replicate autonomously within the host bacterium, but several steps in replication rely on host-encoded factors, making the compatibility between the plasmid and the host essential for maintenance of the plasmid. On the other hand, plasmids are not essential to the host. In fact some small plasmids are cryptic i.e. they have no assigned function besides their own maintenance. However, plasmids often contain genes coding for trades that can be advantageous or even essential for survival in certain environments such as resistance to antibiotics, phages and heavy metals, bacteriocin production, metabolism of carbohydrates etc. (Pouwels and Leer 1993;Wang and Lee 1997).

In lactobacilli native plasmids are prevalent. At least 25 different species of lactobacilli have been shown to harbour natural resident plasmids and multiple plasmids (usually from 1-10) have commonly been identified in a single strain (Wang and Lee 1997). Most plasmids in lactobacilli are small (<10 kb) cryptic plasmids, however, larger plasmids of more than 100 kb have also been detected (Pouwels and Leer 1993;Wang and Lee 1997).

3.2 Plasmid transfer by conjugation

Transmissible plasmids can be either conjugative meaning that they encode a self-sufficient transfer system or mobilizable implying a requirement for supplementary conjugative genes to accomplish transfer (Francia et al. 2004). Mobilizable plasmids commonly harbour a mobilization region, which contain genes necessary for relaxation of the DNA (*mob*) and an origin of transfer (*oriT*). In contrast to conjugative plasmids, mobilizable plasmids lack (*tra*) genes responsible for creation of mating-pair formation (Francia et al. 2004). Transfer of mobilizable plasmids is therefore dependent on the presence of *tra* genes as described above. If transfer is mediated by another conjugative (helper) plasmid, the mobilizable plasmid may either be co-transferred with the helper plasmid (conduction) or transferred alone to the recipient (donation). The further dissemination of the

mobilizable plasmid from the new host may therefore be restricted if no operational *tra* genes are available in the new host.

Plasmid DNA relaxases are thought to be evolutionary related amongst most transferable plasmids and the phylogenetic relatedness has been used as a criterion for classification into different relaxase families and small mobilizable plasmid families (Francia et al. 2004;Grohmann et al. 2003). The plasmid relaxases are key components required to prepare the DNA for transfer. By binding to the supercoiled plasmid DNA and catalysation of a transesterification reaction, the DNA is nicked in a strand-specific manner at *oriT*. The resulting product is a cleaved double-stranded DNA molecule with a free 3'-OH end and the relaxase covalently bound to the 5' end of the strand via a phosphodiester bond (Fig. 2). In order to produce the single strand of plasmid DNA that can be transferred to the recipient, the interaction of a DNA helicase to unwind the intermediate DNA molecule is required. The helicase activity is incorporated in some plasmid relaxases, however in others the source of activity has not been established (Byrd and Matson 1997).

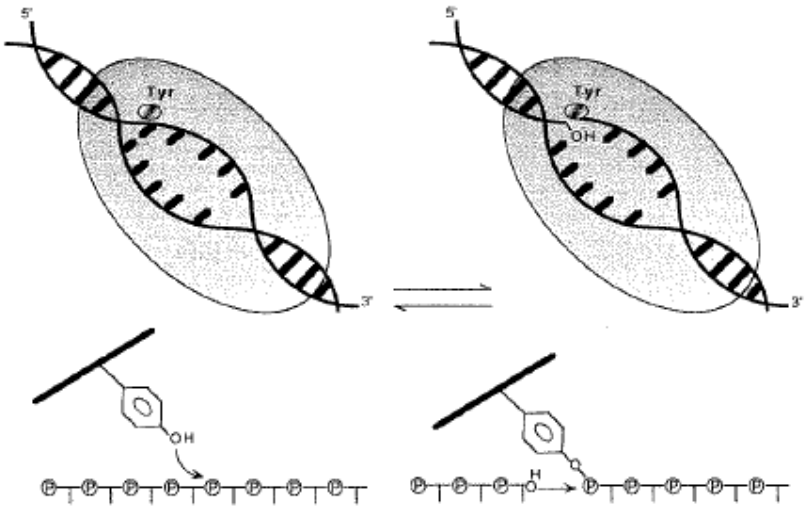

Fig. 2: Activity of a DNA relaxase. Nicking of supercoiled plasmid DNA at *oriT* by attack of the active tyrosine residue and covalent binding of the relaxase to the 5'end of the cleaved DNA. Adapted from (Byrd and Matson 1997).

The relaxase enzymes contain three conserved motifs forming part of the catalytic centre. Motifs I and III have been universally found in relaxases of both conjugative and mobilizable plasmid origin. Motif I contains the active tyrosine residue, which is involved in the nucleophilic attack and nicking at *oriT* and motif III (characterized by two conserved histidine residues) has been proposed

to support the attack by the active tyrosine residue by binding of the Mg^{2+} or Mn^{2+} cation required for the cleavage reaction (Grohmann et al. 2003).

In contrast to *mob* and *tra* genes, which can be provided in *trans*, the only element required in *cis* for a plasmid to be transferable is the *oriT*. However, different families of *oriT* exist and successful transfer is dependent on the ability of the relaxase to recognize *oriT* but also on compatibility between the *mob* and *tra* genes (Grohmann et al. 2003).

3.3 Mechanisms of replication

The two major mechanisms of plasmid replication are the theta type replication and rolling-circle replication (RCR). The theta mechanism is so named due to the characteristic theta shape that coupling between the newly synthesized and the parental plasmid strands assume during replication. Replication is initiated at either one or several origins by dispersion of the parental strands followed by synthesis of a primer RNA, which is covalently extended by DNA polymerase. The leading- and lagging-strand are synthesized simultaneously and can be either in the same direction or in opposite directions, but whereas replication of the leading strand is continuous synthesis of the lagging strand are discontinuous, requiring the synthesis of primer RNAs at regular intervals (del Solar et al. 1998). Theta replication is prevalent amongst larger Gram-positive plasmids such as those belonging to the pAMβ1 family of plasmids isolated from various enterococci and streptococci (Bruand et al. 1993). However, in small Gram-positive plasmids, the general mode of replication is RCR. Since focus during this Ph.D-project primarily has been on plasmids replicating via RCR, the following paragraph will be devoted to this mechanism.

Initially, the mechanism was identified in a number of small (< 10 kb), multicopy plasmids from Gram-positive bacteria and indeed most RCR plasmids belong to this category. However later, RCR has also been detected amongst plasmids of Gram-negative origin (Actis et al. 1999). At least fourteen families of bacterial RCR plasmids have been classified according to homology in factors crucial for their replication namely the replication initiator (Rep) protein and the origin of replication (*ori*) also denoted the double-strand origin (*dso*) (Ilyina and Koonin 1992;Khan 1997;Osborn 2008). Most RCR plasmids from *Lactobacillus*, however seem to belong to RCR group II or III (Table 5), having the RCR plasmids pMV158 originating from *Streptococcus* and pC194 originating from *Staphylococcus*, respectively, as prototypes (Khan 1997).

Table 5: Sequenced RCR plasmids from *Lactobacillus* divided into RCR groups according to similarities in Rep proteins according to (Osborn 2008). Special features concerning antibiotic resistance genes or mobilization (*mob*) genes are listed when found.

Name	Size (bp)	Host species	RCR group	Special features	Accession No. (Reference)
PLFE1	4031	*L. plantarum*	II	*mob*[1]/*erm*	This study
pLC2	2489	*L. curvatus*	II		Z14234 (Klein et al. 1993)
pA1	2820	*L. plantarum*	II	*mob*[2]	Z11717 (Vujcic and Topisirovic 1993)
pLA106	2862	*L. acidophilus*	II	*mob*	D88438 (Sano et al. 1997)
pLB4	3547	*L. plantarum*	II		M33531 (Bates and Gilbert 1989)
pLF1311	2389	*L. fermentum*	II		X74860 (Aleshin et al. 1999)
pLH2	5727	*L. helveticus*	II		X81981 (Pridmore et al. 1994)
ppsc22*		*L. plantarum*	II		X95843 (Cocconcelli et al. 1996)
pLEM5**	3382	*L. fermentum*	III	*erm*	U48430 (Fons et al. 1997)
pC30il	2140	*L. plantarum*	III		JO3319 (Skaugen 1989)
pGT232	5113	*L. reuteri*	III		U21859 (Heng et al. 1999)
pLAB1000	3331	*L. hilgardii*	III	*mob*	M55222 (Josson et al. 1990)
pLC88	3501	*L. casei*	III	*mob*	U31333 (not published)
P353-2	2425	*L. pentosus*	III		X62347 (Leer et al. 1992)
pLP1	2093	*L. plantarum*	III		M31223 (Bouia et al. 1989)
pLTK2	2295	*L. plantarum*	III		AB024514 (Kaneko et al. 2000)
pTC82***		*L. reuteri*	III	*cat*-TC	AF183511 (Lin et al. 1996;Lin et al. 2001)

*vector derived from a 7 kb plasmid (only the replication region has been sequenced). **vector derived from a 5.7 kb plasmid, pLEM3. ***Only the replication region and the antibiotic resistance gene have been sequenced from plasmid pTC82. [1]Putative truncated *mob* gene - no expression observed. [2]Putative truncated mob gene, presumably encoding a non-functional protein. *Erm*, erythromycin resistance gene; *cat*-TC, chloramphenicol acetyltransferase.

The RCR plasmids have a common mode of replication (Fig. 3), although variations in the individual steps may exist. In general, replication is initiated by interaction between the Rep protein and its cognate *dso.* This interaction results in a structural rearrangement of the *dso* region, which commonly exposes the nick site within the loop of a hairpin structure (Fig. 3B) (Khan 1997). Hereafter, the Rep protein cleaves the leading strand at the nick site and binds covalently to the 5' phosphate end of the DNA. A 3' OH end is thereby left free to function as a primer for replication of the new leading-strand (Fig. 3C). The next extension step involves several host proteins including DNA helicase that unwinds the double-stranded plasmid DNA making it accessible for replication by DNA polymerase III. The polymerase extends the replication fork unidirectionally, synthesizing a new leading strand and simultaneously replacing the parental leading strand, which subsequently are coated by the single-stranded DNA binding (SSB) protein (Fig. 3D). Upon accomplishment of one circle, i.e. approximately 10 nucleotides beyond the regenerated *dso* nick

site, the synthesis is terminated by the second monomer of the Rep protein, which nicks and thus releases the displaced strand. The result of one round of replication is a double-stranded DNA molecule constituted by the parental lagging-strand and the newly synthesised leading-strand and a single-strand intermediate consisting of the parental leading-strand (Fig. 3E).

The Rep protein normally supports only one round of plasmid replication, which is important for regulation of the plasmid replication. Cessation of replication often occurs by inactivation of the Rep protein through attachment of an oligonucleotide (approximately 10bp) to its active tyrosine residue. This oligonucleotide corresponds to the sequence immediately downstream of the nick site (Fig. 3F) (Khan 1997;Khan 2005).

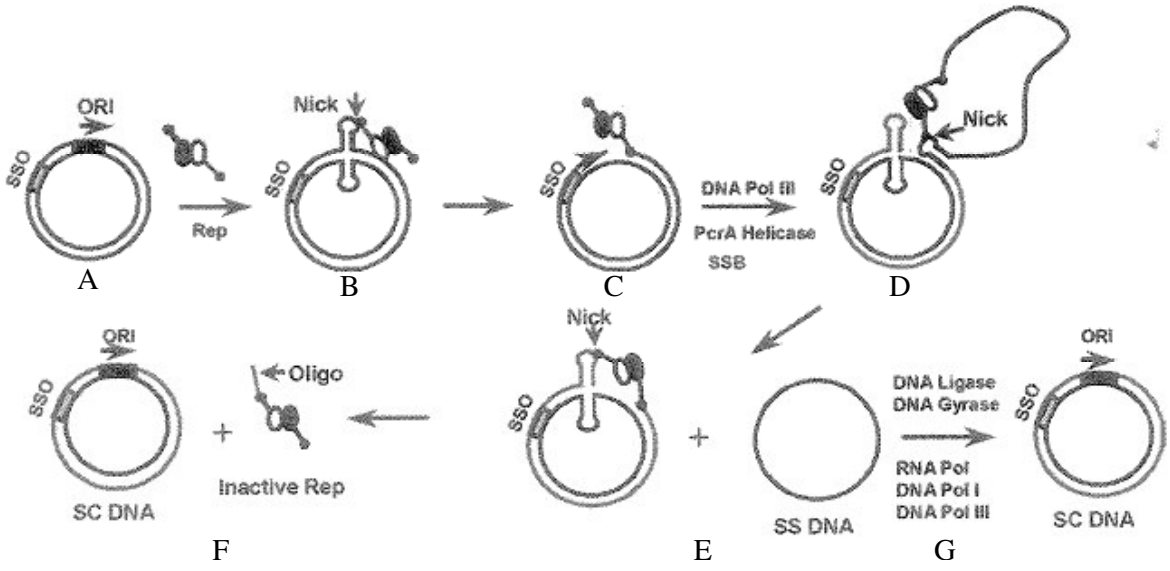

Fig. 3: Model for replication of rolling-circle plasmids. SC, supercoiled; SS, single-stranded; PcrA Helicase, Gram-positive helicase. Adapted from (Khan 2005).

All RCR plasmid families replicate through production of single-strand intermediates and detection of single-stranded DNA have therefore commonly been used as verification of the RCR mechanism (Josson et al. 1990;Leer et al. 1992;Vujcic and Topisirovic 1993;Wu et al. 2007). However, completion of plasmid duplication requires conversion of the single-strand intermediate into double-stranded DNA. The single-strand origin (*sso*) is the starting point of lagging-strand synthesis and lack of a functional *sso* has been shown to result in accumulation of single-stranded DNA (Leer et al. 1992;van der Lelie et al. 1989) and thus segregational instability of the plasmid (Gruss and Ehrlich 1989). The presence of several functional *sso*s on a plasmid is possible (van der Lelie et al.

1989) but in most cases a single *sso* is placed immediately upstream the *dso*. Upon completion of the leading-strand synthesis, the *sso* is present in a single-stranded form and the *sso* promoter sequence can be recognized by host RNA polymerase. Hereby a short primer RNA is synthesized and used by DNA polymerase to initiate replication of the lagging strand using the leading-strand as a template (Fig. 3G) (Kramer et al. 1999).

3.4 Copy number control

Plasmids can range in copy number from one to several hundred per cell, but for a given plasmid replicating under constant conditions, the copy number is relatively invariable. Partitioning systems control the equal distribution of plasmid copies from the mother cell to the two daughter cells. In plasmids that are only present in one or a few copies, an effective partitioning system is very important to ensure that all dividing cells receive the plasmid. In contrast, multicopy plasmids does not always require such a system, since by chance the risk of a daughter cell receiving no copies is somewhat smaller (Novick 1987). As a consequence, RCR plasmids typically do not contain partitioning systems but instead control copy number by regulation of replication (Rasooly and Rasooly 1997).

Control of plasmid replication is a critical point in maintaining copy number. In general, two types of control systems exist for replication. One mechanism is based on repeated DNA sequences (i.e. iterons) located at different sites of the plasmid (Actis et al. 1999). Several mechanisms act in concert to control the copy number in iteron-based regulation. The active Rep protein concentration is limited in several ways, e.g. by binding of the protein to iterons. However, the limitation of active Rep protein only works as a buffer, but is not sufficient to control fluctuations in plasmid copy number. The most important regulator in this system is inactivation of the origin of replication (*ori*). A package of iterons is positioned adjacent to or overlapping with *ori*. If the plasmid copy number is increased above the normal level or if another plasmid harbouring the same replication control is introduced into the same host, the elevated plasmid concentration will be counterbalanced by increased pairing of iterons belonging to separate *ori*s. This pairing is believed to cause a steric hindrance to their function (Paulsson and Chattoraj 2006).

The other basic control mechanism in plasmids relies on small complementary RNA molecules known as antisense or countertranscript (ct) RNAs. These molecules act either by hybridizing to a plasmid-determined target that inhibits replication directly or they act indirectly by hindering synthesis of the Rep protein (Actis et al. 1999).

In contrast to the iteron-based system, the limitation of Rep protein achieved in this way is sufficient to control the plasmid copy number. However, in some plasmids such as RCR plasmids belonging to the pMV158 family, ctRNAs acts in concert with proteins to control plasmid replication (del Solar and Espinosa 1992). In the pMV158 derivative plasmid pLS1, synthesis of the replication initiation protein RepB is controlled both by a small 50-nucleotide long ctRNA and CopG (previously denoted RepA) (Fig. 4). ctRNA is transcribed from promoter P_{ct} in the opposite direction of the bicistronic mRNA encoding CopG and RepB. ctRNA is believed to control synthesis of RepB at the translational level by pairing with a region of the *copG-repB* mRNA including the RBS of *repB* and thus inhibit binding of the ribosomes (del Solar et al. 1997). CopG exerts its controlling effect by binding to a region encompassing the –35 promoter box of the single promoter P_{cr}, responsible for transcription of the operon containing *copG* itself as well as *repB.* Hence, CopG acts as a transcriptional repressor protein preventing binding of host RNA polymerase and thus synthesis of *copG-repB* mRNA (del Solar et al. 2002).

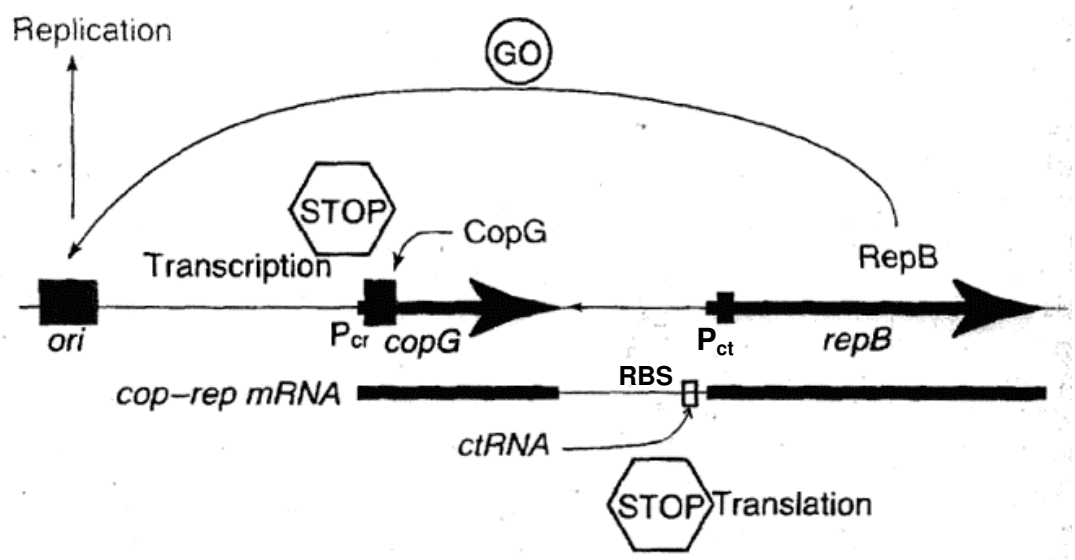

Fig. 4: Copy control regulation in pLS1. RepB exerts a positive effect by initiating replication of pLS1. CopG and ctRNA are negative regulators of RepB (see text for details). Modified from (Rasooly and Rasooly 1997) in accordance with (del Solar and Espinosa 1992).

3.5 Plasmid incompatibility

Plasmid incompatibility is when two plasmids fail to be stably maintained in the same host in the absence of selective pressure. This phenomenon is the result of plasmids harbouring similar functions for inheritance such as partition or replication (Novick 1987). As these functions are very fundamental for the plasmid, classing the plasmids into incompatibility groups is used as a measure of relatedness, with plasmids belonging to the same group sharing common features (Actis et al. 1999). The traditional method of incompatibility grouping relies on the simple observation of elimination or coexistence between different plasmids transformed into the same cell line. However, a single base change in specific regions covering these functions can render two otherwise incompatible plasmids compatible (Couturier et al. 1988). Therefore, this method can potentially place two plasmids into different incompatibility groups despite a remarkable degree of affiliation. In contrast, two plasmids may be assigned to different incompatibility groups although they share similar replication functions. Hence, if one of the plasmids carry more than one region of replication it may avoid segregation by switching to control by the compatible mechanism and thereby conceal the presence of the former (Couturier et al. 1988). Concurrently with the improved accessibility to DNA sequencing data, a more comprehensive and rapid classification scheme has been made based on similarity of replication (*rep*) region DNA sequences. Classification of plasmids using this criteria can be made with variable accuracy depending on the choice of method e.g. sequence analysis or hybridization using more or less degenerated probes and stringency conditions (Couturier et al. 1988).

3.6 Host-range

The host range of plasmids is highly variable with some plasmids only replicating in hosts belonging to a few species thus exercising a narrow host-range and other plasmids showing a broad host-range proliferating in a variety of different genera or even across kingdoms. The majority of plasmids are however confined to either Gram-positive or Gram-negative bacteria (Courvalin 1994).

Many traits are decisive of the host-range of a conjugative or mobilizable plasmid and for a given plasmid the specific factors are often poorly understood. Inheritance of a plasmid in a new host requires successful accomplishment of two principal steps; first the transfer process itself should be possible and subsequently, stable replication in the recipient must occur. The former step is generally regarded as less prohibitive and the latter step rate-limiting (del Solar et al. 1996).

The key component responsible for establishment of the plasmid in the host is the replicon, which can be more or less autonomous but often must comply with certain host factors to accomplish replication. In the laboratory, fusions of replicons functional in different hosts are used to increase the diversity of hosts fitted for a given plasmid. However, in nature this strategy does not seem feasible and several plasmids containing multiple replicons though exhibiting a narrow host-range have been described (del Solar et al. 1996).

The host-range of RCR plasmids also extents from narrow to extremely broad. An example of the latter is the mobilizable plasmid pIP823 from *L. monocytogenes.* Mobilization of pIP823 has been reported to occur by the aid of self-transferable conjugative plasmids to strains of *E. faecalis*, *S. aureus*, *B. subtilis* and *E. coli* (Charpentier et al. 1999). In RCR plasmids, synthesis of the leading strand is independent of host proteins, but initiation of lagging strand replication requires recognition of the *sso* by host RNA polymerase. Four types of *sso*s have been described in the litterature namely *sso*A, *sso*T, *sso*U and *sso*W. Most of the *sso*s function only optimally in their natural hosts, however the *sso*U has the quality that it can be efficiently recognized in a broad range of Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Lactococcus lactis*. The ability of the host RNA polymerase to bind efficiently to the promoter sequences of the *sso* has been proposed to be an important determinant of the host-range of the plasmid (Kramer et al. 1999).

In the pheromone-inducible plasmids, which are present in *E. faecalis*, release of pheromones trigger bacterial clumping and thus promote cell-to-cell contact (Dunny et al. 1995). However, in most other cases, the factors controlling establishment of cell-to-cell contact between potential donor and recipient bacteria remain cryptic. An aspect that influences the success of mating-pair formation is the interaction between the pilus/pore junction and the lipopolysaccharides and surface membrane proteins of the recipient cell (Thomas and Nielsen 2005). In this regard, the cell wall of Gram-positive bacteria has been suggested to constitute an important barrier to transfer of DNA from *E. coli* (Courvalin 1994). Though, speaking in favour of a relatively unrestrictive transfer barrier between Gram-negative and Gram-positive bacteria, a recent study showed dissemination of an IncP-1 plasmid from *Pseudomonas putida* to a broad range of recipients including a Gram-positive member of the *Actinobacteria* (Musovic et al. 2006). However, the collection of transconjugants was performed in a cultivation-independent manner. Thus the study merely verified the ability of the plasmid to be transferred to, but not maintained in the recipient bacteria.

Another factor that may affect the dissemination probabilities of a given plasmid is the host in which it resides. A study evaluating the diversity of transconjugants receiving the IncP-1β plasmid pB10 showed a significant difference depending on the donor, when the plasmid was mated to the same population of sludge bacteria (De et al. 2005). Obviously, this finding is not a matter of divergence in integration or replication potential of the plasmid in the various recipients but sooner a result of attachment and establishment of well-functioning mating channels between different mating pairs. A side-issue to this is also the environmental preferences of certain bacteria. Hence some bacteria may be more prone to occupy a distinct niche (for instance in the GI tract) and thus encounter another variety of potential recipients than the donor residing in a different niche. All in all, a plasmid may be disseminated in promiscuous ways and a seemingly "dead-end" of transfer, may be overcome by movement to a new host, facilitating the apparently insuperable move.

4. Models of the human gastrointestinal tract

4.1 Factors affecting transfer in the gastrointestinal tract

An intimate contact between the donor and recipient bacteria, is as earlier mentioned a prerequisite for exchange of antibiotic resistance genes by conjugation. In the GI tract, the density of bacteria increases from the small intestine consisting of duodenum, jejunum and ileum and downwards to the caecum, which is placed as a blind pocket at the connection between the small and large intestine, to the colon, comprising the large intestine (Fig. 5). A relatively low concentration of approximately 10^3-10^4 cfu/g is present in duodenum, constituting the upper part of the small intestine whereas in the colon an exceptionally high concentration of 10^{11}-10^{12} cfu/g of intestinal contents is found. In the colon, bacterial mass has been estimated to account for up to half of the volume content (Isolauri et al. 2004). This large density of bacteria means a great potential to encounter recipients and establish cell-to-cell contact.

The GI tract contains from 300 to 500 different bacterial species that may be either transiently present or comprise a part of the indigenous microbiota (Guarner and Malagelada 2003). The bacteria are not equally distributed throughout the intestine since environmental conditions differ between the intestinal compartments, but also within small-scale distances of compartments (such as between the mucus layer and lumen content), conditions and thus bacterial compositions are varying (Anderson 2003). In the small intestine, *Lactobacillus* counts for a significantly large

proportion of bacteria, as is shown in Fig. 5, depicting the numerically dominant genera in each intestinal compartment.

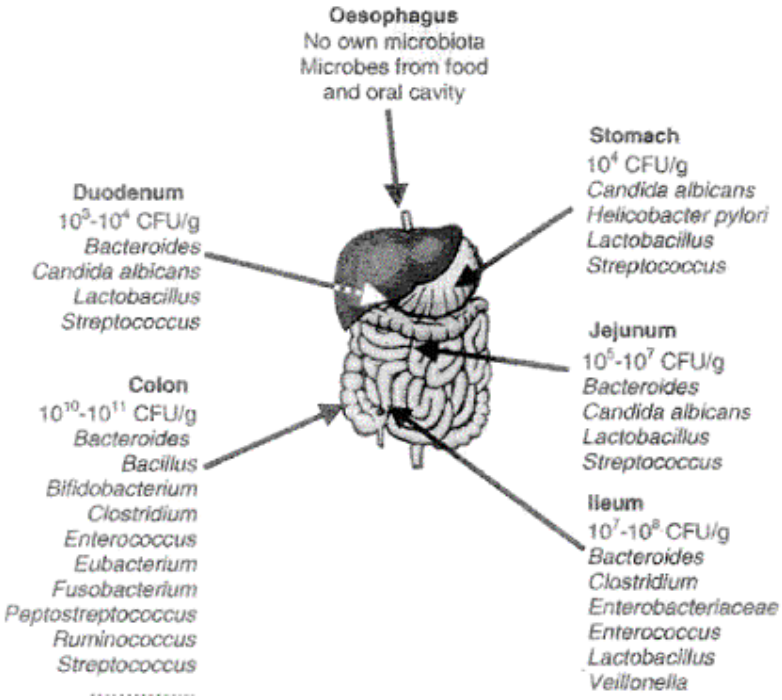

Fig. 5: Bacterial density and composition of numerically dominant genera in the adult human GI tract. Adapted from (Isolauri et al. 2004)

When a bacterium enters the GI tract via food etc., it encounters a very hostile environment with acidity as low as pH 2 in the stomach and bile acids excreted in the small intestine. Furthermore, release of inhibiting factors from other bacteria and competition for nutrients and available space (i.e. the colonization resistance of the indigenous microbiota) may affect the physiological status of the ingested bacteria. In order to colonize the GI tract, these obstacles have to be overcome by growth and/or binding to intestinal surfaces at an efficiency that counteracts the peristaltic movements of the intestine. In this regard, bacterial ability to initiate mating-pair formation has been suggested to influence their survivability. Apart from the obvious potential in adaptability by accepting genetic elements, also attachment to intestinal epithelial cells and surface structures has been proposed as a benefit of the conjugation system. In some cases the presence of pili or surface aggregation substances that are required for conjugation, may enable bacteria to hold on to intestinal surfaces or to each other, thus promoting colonization (Licht and Wilcks 2005).

Although the physiological status of the donor and recipient bacteria influences their ability to transfer and receive plasmids, colonization of the gut is not a prerequisite for transfer of antibiotic resistance genes. Hence, several studies have reported transfer from a donor, which was only transiently passing the intestine (Gruzza et al. 1994;Licht et al. 2002;Schlundt et al. 1994). Yet, colonization may increase the chance of mating events in the intestine both due to proliferation and potentially higher numbers of the donor, but also to increase of the time span in which potential contacts may take place. The latter may especially be important upon interruptions of environmental conditions in the GI tract, for instance imposed by antibiotic treatment.

4.2 *In vitro* mating

In vitro mating is the most common method to investigate the conjugation ability of specific donor and recipient bacteria and an easy way to transfer specific desirable mobile plasmids to new recipients. This method traditionally involves separate culturing of the test bacteria followed by mixing in broth media or on agar plates (with or without filters). The mixture are incubated for a given period allowing the mating process to occur and thereafter harvesting and plating on selective media for both the recipient, donor and transconjugant bacteria are performed. This method is cheap, easy and fast compared to *in vivo* experiments and allows the conjugation to proceed under controlled conditions. The mating procedure can be optimized to offer the best conditions for transfer and the impact of specific factors on transfer frequency can be analysed. Furthermore, the transfer potential of different mobile elements and mating-pairs can be compared under similar conditions.

A draw-back of the method is absence of standardization and thus comparability between different published experiments, and also lack of knowledge about conditions optimal for transfer. Several factors affect transfer, amongst others the growth rate and induction of different stress response mechanisms of the donor and recipient bacteria (Mcmahon et al. 2007). Likewise, the phase of the mating medium e.g. if it is solid or liquid (Wilcks et al. 2005) and the structure of the mating surface, which can be prepared *in vitro* with filters of different pore sizes (Gevers et al. 2003b;Sasaki et al. 1988), have been shown to change transfer frequencies or reverse positive or negative outcomes. However, the optimal transfer conditions for a given mobile element and mating-pair may not necessarily be ubiquitous for all mating-pairs and even opposite effects achieved by regulation of a specific factor may occur.

In conclusion, *in vitro* mating can be used to assess if a specific genetic element have the potential to be transferred. However, negative conjugation results *in vitro* do not justify rejection of the possibility that transfer can take place under different circumstances *in vivo.* Furthermore, the effectiveness of *in vivo* transfer for a given mobile element and mating-pair is difficult to predict by *in vitro* analyses. In contrast to *in vivo* studies, *in vitro* mating enables calculation of the number of transfer events. In the intestinal environment this calculation is severely biased by growth of the bacteria and thus vertical transfer of the mobile element. Several studies have reported that the *in vivo* transfer potential deviated considerably from the predicted potential based on *in vitro* mating frequencies (Dahl et al. 2007;Netherwood et al. 1999).

4.3 Germ-free/di-associated rats

In this project germ-free rats have been used as an *in vivo* model for the human GI tract. This model constitutes a relatively controlled approach to evaluate the ecology of bacteria during physical influences resembling those encountered in human. A remarkable advantage of germ-free rats is the ability to possess full control of bacteria added to the "test system". However, in contrast to human, the rat is coprophagues meaning that it ingests faeces. When investigating bacterial interactions with this model, practical measures such as gratings and individual cages therefore need to be considered in order to prevent "re-inoculation" of the rats.

Basically, the GI system of the rat and human is similar, nevertheless variations in gut physiology, pH, peristalsis, secretions etc. may result in different bacteria being promoted in the two hosts (Rumney and Rowland 1992). The same applies to differences in the food/feed. Though, if this is considered important for a particular experiment, a specific diet can be prepared to the test animals. Ethical considerations obviously need to be taken when planning experiments with research animals such as germ-free rats. Whenever possible *in vitro* methods should replace animal experiments yet, if the alternative is experiments conducted with human volunteers subjected to an increased risk, germ-free rats may be preferred.

Germ-free rats allow for determination of the gene transfer potential of specific donor-recipient pairs without competition and interference from other intestinal bacteria. By di-association i.e. inoculation with only two different strains of bacteria, a much higher concentration of the particular bacteria is achieved than would be the case in a normal intestine harbouring a complex intestinal microbiota conferring colonization resistance. The bacteria are to a high degree exposed to the same factors as they are during passage of a normal GI tract however, their higher concentration increases

the chances of mating-pair formation and concurrently lowers the limit of detection for gene transfer (Alpert et al. 2003;Doucet-Populaire et al. 1992). This also implies that the di-associated germ-free rat model overestimates the transfer frequency compared to a normal GI tract though, it predicts the possibility of transfer taking place *per se*. Di-associated germ-free rats have been designated a worst-case *in vivo* model for gene transfer, however, by using a full-microbiota model the potential of antibiotic resistance transfer may be overlooked.

4.4 Human microbiota-associated rats

Germ-free rats constitute the starting point for development of several different models. The di-associated rat constitute one of the most simple models, however, gradually more complex models can be made by inoculation with several different bacterial species that can be specifically chosen and carefully composed or be a more or less unspecified microbiota derived from human fecal samples for development of the human microbiota-associated (HMA) model.

In the HMA model, germ-free animals receive a single inoculation with a complex human microbiota and thereafter, gradual selection of the microbiota takes place to suit the environment in the new host (Bernbom et al. 2006;Licht et al. 2007). Standardisation of the time after inoculation and until initiation of the experiment is therefore desirable to minimize variation between experiments (Bernbom et al. 2006). This time period should be a compromise between least development in the microbiota composition during the experiment and still a good resemblance with the originally inoculated microbiota composition.

The physiology and feed of the rat may as earlier mentioned favour colonization of certain and other species than in the human though, the composition of bacteria as a starting point will be similar and only their relative concentration will be changed in the model compared to the human inoculation material. An exact comparison of the inoculation material and the bacteria selected for in the rat is difficult to obtain, since the human microbiota to a large degree consists of non-culturable and uncharacterized bacteria. Several studies have been performed, applying various methods involving cultivation on selective media, PCR-based amplification and fingerprinting assays as well as sequencing and identification of specific species etc. A recent study compared profiles of rat faecal samples taken two weeks after dosage of a human faecal inoculum and the corresponding inoculum (Licht et al. 2007). Denaturing gradient gel electrophoresis (DGGE) on PCR amplified 16S rRNA genes and subsequent excision, cloning and sequencing of obtained bands were performed, and the results suggested a composition of dominant species that largely

were the same in the two hosts. In contrast, a study of HMA mice analysed with terminal restriction fragment length polymorphism (T-RFLP) up till eight weeks after administration of inoculum concluded that a significant selection occurred in the mice and that the dominant bacterial groups therefore varied from those in the inoculum (Kibe et al. 2005).

Bernbom and coworkers investigated the effect of time on the composition of faecal microbiota of HMA rats after inoculation (Bernbom et al. 2006). They reported a similarity of almost 80% when comparing the composition of individual animals one weak and four weeks after inoculation, using DGGE and T-RFLP on samples amplified by PCR with universal primers. Analysis of the variation between rats inoculated with the same faecal material showed similarities of 50% and 63% for DGGE and T-RFLP, respectively, at all time points during the experiment. The latter comparison also revealed clustering between animals belonging to the same gender i.e. between male and female rats. Faster peristalsis and thus transit times in the larger males was suggested to exert these variations in colonization pattern however, other genetic differences could also be influential (Bernbom et al. 2006).

In conclusion, the HMA model is subject to selection at some degree and there may be differences between individuals, gender and time after inoculation, but also age of the animals and animal species etc. Some of these factors can be accounted for in the experimental set-up, whereas others are implicit variations. Despite this, I think that the HMA model has many applications to investigate complex bacterial ecology in an environment that resembles the human GI tract. The model also has the advantage that several rats can be inoculated with the same starting material. Compared to experiments performed with human volunteers where large differences between individuals may blur the results, variations between rats may be small and fewer “replicates” therefore needed to determine statistic significant results.

4.5 Antibiotic-treated animals

Antibiotic-treated animals such as rats and mice are other commonly used models of the human GI tract. Treatment with antibiotics such as streptomycin disturbs both aerobic and anaerobic components of the microbiota, which in turn affects parameters such as pH (increasing) and the concentration of volatile fatty acids (decreasing) in intestinal contents (Que et al. 1986). The colonization barrier is thus impaired compared to a full microbiota model and chances of the chosen test bacteria colonizing the GI tract are concurrently improved.

Typical use of the antibiotic-treated animal model includes treatment of the animals immediately prior to inoculation with the test bacteria and sometimes also during the experiment. A requirement of the test bacteria is therefore that they are either intrinsic resistant or made resistant to the respective antibiotics. Compared to the HMA model, which also have a high colonisation barrier and a more realistic composition of the microbiota to simulate that in the human GI tract, the antibiotic-treated animals have a more "mature" microbiota. The microbiota is inherited to fit the animal, which may be important for the authenticity of the model in relation to colonisation of the intestinal microniches. In antibiotic-treated animals, the intestine has gone through a realistic colonisation phase where probably all available niches are occupied by the bacteria best adapted for these. In contrast, the bacteria occupying the corresponding niches in the HMA model have not endured the same selection and may not comprise an equivalently strong colonisation barrier.

5. Results

Paper I

Louise Jacobsen, Andrea Wilcks, Karin Hammer, Geert Huys, Dirk Gevers and Sigrid Rita Andersen (2007). Horizontal transfer of *tet*(M) and *erm*(B) resistance plasmids from food strains of *Lactobacillus plantarum* to *Enterococcus faecalis* in the gastrointestinal tract of gnotobiotic rats. FEMS Microbiology Ecology 59, pp. 158-66.

Horizontal transfer of *tet*(M) and *erm*(B) resistance plasmids from food strains of *Lactobacillus plantarum* to *Enterococcus faecalis* JH2-2 in the gastrointestinal tract of gnotobiotic rats

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Lactobacillus plantarum; antibiotic resistance; horizontal gene transfer; gnotobiotic rats; gastrointestinal tract.

Abstract

Two wild-type strains of *Lactobacillus plantarum* previously isolated from fermented dry sausages were analysed for their ability to transfer antibiotic resistance plasmids in the gastrointestinal tract. For this purpose, we used gnotobiotic rats as an *in vivo* model. Rats were initially inoculated with the recipient *Enterococcus faecalis* JH2-2 at a concentration of 10^{10} CFU mL^{-1}. After a week, either of the two donors *L. plantarum* DG 522 (harbouring a *tet*(M)-containing plasmid of *c.* 40 kb) or *L. plantarum* DG 507 [harbouring a *tet*(M)-containing plasmid of *c.* 10 kb and an *erm*(B)-containing plasmid of *c.* 8.5 kb] was introduced at concentrations in the range of 10^{8}–10^{10} CFU mL^{-1}. Two days after donor introduction, the first transconjugants (TCs) were detected in faecal samples. The detected numbers of *tet*(M)-TCs were comparable for the two donors. In both cases, this number increased to *c.* 5×10^{2} CFU g^{-1} faeces towards the end of the experiment. For *erm*(B)-TCs, the number was significantly higher and increased to *c.* 10^{3} CFU g^{-1} faeces. To our knowledge, this is the first study showing *in vivo* transfer of wild-type antibiotic resistance plasmids from *L. plantarum* to *E. faecalis*.

Introduction

The major financial and societal costs caused by the emergence and evolution of antibiotic resistance in pathogenic bacteria represent a well-known problem. The attenuation of this problem is complicated by commensal bacteria that may act as reservoirs for antibiotic resistance determinants found in pathogens (Salyers & Shoemaker, 1996; Levy & Marshall, 2004). This statement is supported by the fact that the same type of genes encoding resistance to, for example, tetracycline, erythromycin, chloramphenicol, streptomycin and streptogramin have been found in commensal lactococci and lactobacilli as well as in potentially pathogenic enterococci and pathogenic streptococci (Teuber *et al.*, 1999). A remarkable similarity in resistance genes has also been observed for tetracycline-resistant *Lactobacillus plantarum*, *Lactobacillus sakei* ssp. *carnosus*, *L. sakei* ssp. *sakei*, *Lactobacillus curvatus* and *Lactobacillus alimentarius*, all of which have been previously isolated from Belgian fermented dry sausages (Gevers *et al.*, 2000, 2003a). Partial sequencing of the tetracycline resistance genes detected in all these species revealed two sequence homology groups (SHGs) with $\geq$99.6% identity to *tet*(M) genes of *Enterococcus faecalis* and *Neisseria meningitidis* (SHG I) and of *Staphylococcus aureus* (SHG II) (Gevers *et al.*, 2003a; Huys *et al.*, 2004).

Lactobacilli are generally recognized as safe, and are industrially important food-grade organisms used as probiotics and starter cultures in fermented foods. They are present in ready-to-eat foods and are also indigenous members of the human intestinal microbiota. Data on antibiotic resistance in lactobacilli are relatively limited. Only recently, a number of studies have recorded antibiotic susceptibility profiles for various *Lactobacillus* species in order to facilitate differentiation of intrinsic resistance from acquired (and thus potentially transferable) resistance

(Charteris *et al.*, 1998; Danielsen & Wind, 2003; Cataloluk & Gogebakan, 2004; Delgado *et al.*, 2005; Florez *et al.*, 2005). Although reports on the presence of antibiotic resistance genes associated with mobile genetic elements are scarce among lactobacilli, the safety implications connected to their presence in organisms for human consumption should be considered (Saarela *et al.*, 2000). Lactobacilli that harbour antibiotic resistance determinants have been found in a broad selection of food products such as fermented drinks and yoghurts (Temmerman *et al.*, 2003), cheese (Herrero *et al.*, 1996; Florez *et al.*, 2005), and meat products (Gevers *et al.*, 2000). Owing to their wide environmental distribution, it is possible that these commensal bacteria act as vectors for the dissemination of antibiotic resistance determinants via the food chain to the consumer, a risk that has so far been poorly addressed.

The aim of this study was to examine the ability of two wild-type *L. plantarum* strains from food origin horizontally to transfer tetracycline and erythromycin resistance genes *in vivo* to the well-documented recipient strain *Enterococcus faecalis* JH2-2. Conjugative dissemination of resistance genes between these strains has been shown previously using an *in vitro* filter mating approach (Gevers *et al.*, 2003b). In order to better represent the natural situation, gnotobiotic rats were used to investigate the potential of the *Lactobacillus* strains to function as vehicles of transferable antibiotic resistance in the gastrointestinal tract.

Materials and methods

Bacterial strains and growth conditions

The two bacterial donors used in this study were *L. plantarum* DG 522 (LMG 21687) and *L. plantarum* DG 507 (LMG 21684) isolated from fermented dry sausages (Gevers *et al.*, 2003a). Strain *L. plantarum* DG 522 contains a *tet*(M) tetracycline resistance gene located on a *c.* 40-kb plasmid (p*tet*(M)$_{DG\ 522}$). Strain *L. plantarum* DG 507 contains a *tet*(M) gene located on a *c.* 10-kb plasmid (p*tet*(M)$_{DG\ 507}$) and an *erm*(B) erythromycin resistance gene located on a *c.* 8.5-kb plasmid (p*erm*(B)$_{DG\ 507}$) (Gevers *et al.*, 2003b). The donor strains were grown at 30 °C in De Man, Rogosa and Sharpe (MRS) medium (Oxoid, Hampshire, UK) containing only tetracycline (*L. plantarum* DG 522) or both tetracycline and erythromycin (*L. plantarum* DG 507) for 24–48 h. The same growth conditions for donor strains were used both for monocultures and for isolation from faecal samples.

Strain *Enterococcus faecalis* JH2-2, showing resistance to rifampicin and fusidic acid (Rif^r, Fus^r) (Jacob & Hobbs, 1974) (LMG 19456), was used as plasmid-free recipient, and was grown in brain heart infusion (BHI) medium (Oxoid) containing rifampicin and fusidic acid for 24 h. The recipient was incubated at 37 °C when grown from monoculture and at 42 °C when isolated from faecal samples.

Transconjugants (TCs) were selected from faecal samples on both BHI and Slanetz & Bartley agar (Oxoid), resulting in similar counts for the two media. For selection of TCs *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$ and *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$ plates were supplemented with rifampicin, fusidic acid and tetracycline, whereas for selecting TCs *E. faecalis* JH2-2,p*erm*(B)$_{DG\ 507}$ plates were supplemented with rifampicin, fusidic acid and erythromycin. Plates were incubated at 42 °C for 48 h. In succeeding analyses, TCs were subcultured in BHI medium with the appropriate antibiotics at 37 °C.

Antibiotics (Sigma, Bornem, Belgium) were used at the following concentrations: rifampicin, 50 μg mL^{-1}; fusidic acid, 25 μg mL^{-1}; tetracycline, 10 μg mL^{-1}; erythromycin, 5 μg mL^{-1}; streptomycin, 500 μg mL^{-1}; and spectinomycin, 500 μg mL^{-1}.

Animal management and experimental design

Six male and six female germ-free Sprague–Dawley rats, originally supplied by Taconic (Germantown, NY) were bred at the Danish Institute for Food and Veterinary Research. The rats had an age of *c.* 3 months at the beginning of the experiment and were housed and fed as previously described (Wilcks *et al.*, 2004). The germ-free status of the rats was verified prior to bacterial dosing by analysing faeces for aerobic and anaerobic growth of bacteria and yeasts. The rats were divided into three groups: (A) five rats receiving *L. plantarum* DG 522 and *E. faecalis* JH2-2, (B) five rats receiving *L. plantarum* DG 507 and *E. faecalis* JH2-2, and (C) two rats receiving only *E. faecalis* JH2-2 as a control.

At day 0, all rats received 1 mL of 10^{10} CFU mL^{-1} of the recipient strain *E. faecalis* JH2-2. The recipient strain was allowed to establish in the rats for 1 week, after which the donor strains were introduced. Each day from day 7 to 10 and 13 to 16 all rats in groups A and B received 1 mL of 10^8–10^{10} CFU mL^{-1} *L. plantarum* DG 522 or *L. plantarum* DG 507, respectively. At days 13–16, 1 μg tetracycline or 1 μg tetracycline +0.5 μg erythromycin was added to the dosing cultures of *L. plantarum* DG 522 and *L. plantarum* DG 507, respectively. During the same period, one rat in control group C was given 1 mL of 1 μg mL^{-1} tetracycline whereas the other received 1 mL of 1 μg mL^{-1} tetracycline +0.5 μg mL^{-1} erythromycin. All bacterial cultures were grown overnight and washed in phosphate-buffered saline (PBS) (Oxoid) before they were given *per os* by gavage after the collection of faecal samples.

Collection and processing of faecal samples

Faecal samples were collected directly from the rats each working day by careful squeezing of the abdomen. Intestinal

samples from duodenum, ileum, caecum and colon were taken at sacrifice. The samples were homogenized by whirly mixing in PBS. Ten-fold dilution series were prepared in PBS and incubated on the appropriate selective agar-plates for enumeration of donors, recipients and TCs as described above. The detection limit for TCs was determined as 2×10^1 CFU g^{-1} faeces.

Verification of TCs by PCR

TCs isolated from the rats were selected on the basis of their phenotypic resistance profile, i.e. Rif^r, Fus^r and Tet^r or Rif^r, Fus^r and Erm^r, by testing their capability of growth on BHI agar supplemented with these antibiotics. In order to verify that these isolates were true TCs and not mutants, PCR assays with primers specific for *E. faecalis* or targetting *tet*(M) and *erm*(B) resistance genes, respectively, were performed. At least five isolates of each TC type (*E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$, *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$ and *E. faecalis* JH2-2,p*erm*(B)$_{DG\ 507}$) were selected from faecal samples of each of the five rats. The isolates were selected from different days during the experiment representative for the period from the first appearance of the TCs until euthanasia. Donor and recipient strains were included as positive and negative controls, respectively.

Bacterial DNA template was prepared by boiling of one colony in 200 μL TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4) for 10 min. After cooling, excess cell material was settled by centrifugation at 5000 ***g*** for 2 min and the supernatant was transferred to new vials and stored at −20 °C until use. PCR reaction mixtures contained 5 μL DNA template, one PuReTaq Ready-To-Go PCR bead (Amersham Biosciences, Buckinghamshire, UK) and 10 pmol of each primer in a total volume of 25 μL. The primers, which were used to check for the presence of *tet*(M) and *erm*(B) genes, were *tet*M-F and *tet*M-R (Warsa *et al.*, 1996; Wilcks *et al.*, 2004) (406 bp) and *erm*B-F and *erm*B-R (Jensen *et al.*, 1999) (424 bp), respectively. *Enterococcus faecalis* species-specific primers were *ddl E. faecalis* E1 and *ddl E. faecalis* E2 (Dutka-Malen *et al.*, 1995) (941 bp).

All PCR amplifications were performed in a PTC-225 Peltier Thermal Cycler (MJ Research, Bio-Rad, Waltham, MA) using the PCR programs as previously described for detection of *tet*(M) and *erm*(B) (Gevers *et al.*, 2003b) and for *E. faecalis* (Dutka-Malen *et al.*, 1995). The PCR products were run on a 1% agarose gel and visualized by ethidium bromide staining.

Determination of minimum inhibitory concentration values

The minimum inhibitory concentrations (MICs) of tetracycline and erythromycin were evaluated by application of an E-test strip (AB Biodisk, Solna, Sweden) on Mueller-Hinton (BBL, Sparks, USA) agar. E-tests were read after overnight incubation at 37 °C for recipients and TCs and at 30 °C for donors.

Plasmid profiles and Southern hybridization

Plasmid DNA was isolated from TCs, donor and recipient strains using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with slight modifications. Exponentially growing cultures were used instead of overnight cultures, and a lysozyme step (2 mg mL^{-1} lysozyme in P1 buffer for 25 min at 37 °C) was included. The plasmid extractions were run on 0.7% agarose gels and the gels were vacuum blotted onto Hybond-N^+ membrane filters (Amersham Biosciences, Little Chalfont, UK). Labelling of DNA probes for *tet*(M) and *erm*(B) with alkaline phosphatase and chemiluminescent detection with CDP-star were carried out using the AlkPhos Direct labelling kit RPN 3690 (Amersham Biosciences) as described by the manufacturer. Amplification products obtained with primer pairs *tet*M-F and *tet*M-R, and *erm*B-F and *erm*B-R were used as probes.

In vitro mating

The ability of the TCs to function as new donors of the *tet*(M) and the *erm*(B) resistance genes was assessed using a filter mating approach. Five isolates of TCs *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$, five isolates of *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$ and five isolates of *E. faecalis* JH2-2,p*erm*(B)$_{DG\ 507}$ obtained from faecal samples and representing different plasmid profiles were evaluated as potential donors. *Enterococcus faecalis* JH2SS resistant to streptomycin and spectinomycin ($Strep^r$, $Spec^r$) (Tomich *et al.*, 1980) isogenic with *E. faecalis* JH2-2 and *E. faecalis* OG1SS ($strep^r$, $spec^r$) (Franke & Clewell, 1981) were used as recipients. As a positive control of the mating conditions, transfer of pAMβ1 from *E. faecalis* JH2-2 [which had received pAMβ1 from *Lactococcus lactis* SH 4174 (Gasson & Davies, 1980)] to *E. faecalis* JH2SS was applied. The mating procedure was as described earlier (Gevers *et al.*, 2003b). In short, exponentially growing donor and recipient cultures were mixed and poured onto a sterile filter (HAWP04700, Millipore, Bedford, MA), and the filters were incubated on non-selective BHI agar plates at 37 °C for 18–20 h. The bacteria were washed off the filters with PBS and appropriate dilutions were spread onto donor-, recipient- and TC-selective agar plates.

Statistics

Reported values are the means from five-fold repetitions. In order to facilitate log transformation of the data for CFU counts, measurements of zero CFU on a plate were set to

1 CFU g^{-1} faeces. Comparison of the *erm*(B)- and *tet*(M)-TC numbers were performed using the Wilcoxon test.

Results

CFU counts of animal samples

In vivo transfer of wild-type plasmids encoding tetracycline and erythromycin resistance was assessed from the donor strains *L. plantarum* DG 522 and *L. plantarum* DG 507 to the recipient *E. faecalis* JH2-2 using germ-free rats. The recipient given to the animals as a single-dose at day 0 colonized readily to attain a stable and high population size of *c.* 5×10^9 CFU g^{-1} faeces throughout the experiment (Figs 1 and 2). The donors were introduced one week after the recipient and were administered daily for two dosing periods each of 4 days (interrupted by a two-day break). The number of donors was lower and less stable than the number of recipients, but remained within the range of 10^5–10^7 CFU g^{-1} faeces (Figs 1 and 2). The fact that the number of donors in the inoculation dose varied might explain the variation in the numbers observed in the animals, because these two numbers seemed to correlate (data not shown). After the first dosing period, the donor strains persisted in the animals at relatively unchanged numbers for 2 days without re-inoculation. Subsequently, the number increased again during the second dosing period, in which sub-therapeutic levels of antibiotics were also added. The sub-therapeutic treatment with antibiotics did not have any detectable effect on the number of recipients, either in the control rats inoculated only with recipients (data not shown) or in the rats inoculated with both recipients and donors (Figs 1 and 2).

The first TCs were detected in faecal samples 2 days after the introduction of either of the donors and increased slightly in number towards the end of the experiment (Figs 1 and 2). During the first 48 h, no bacterial growth was observed on TC-selective agar plates. Throughout the entire transfer experiment, no growth was observed from control rats on plates selective for TCs (data not shown). TCs were detected in all but one rat before the sub-therapeutic treatment with antibiotics was initiated. The development in numbers of TCs selected for resistance to tetracycline *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$ and *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$ was comparable for the two mating pairs and reached a level of *c.* 5×10^2 CFU g^{-1} faeces at the end of the experiment (Figs 1 and 2). For rats inoculated with donor *L. plantarum* DG 507, TCs were additionally selected for erythromycin resistance. The number of *E. faecalis* JH2-2,p*erm*(B)$_{DG\ 522}$ TCs was slightly but significantly ($P < 0.005$) higher than that of the tetracycline-resistant TCs, and reached a level of *c.* 10^3 CFU g^{-1} faeces (Fig. 2).

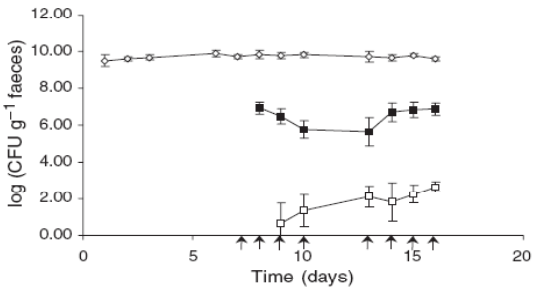

Fig. 1. CFU of the recipient *Enterococcus faecalis* JH2-2 (diamonds), the donor *Lactobacillus plantarum* DG 522 (closed squares) and the trans-conjugants isolated for tetracycline resistance (open squares) from faecal samples. The arrows indicate the time of donor administration. Each point represents the average of data from five rats, and error bars represent standard deviations.

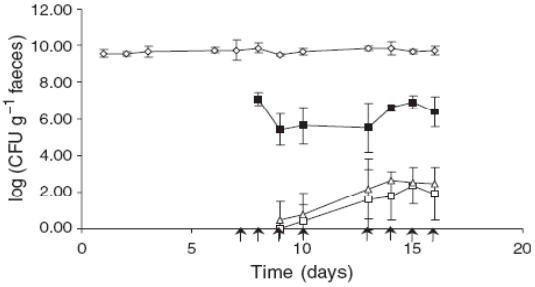

Fig. 2. CFU of the recipient *Enterococcus faecalis* JH2-2 (diamonds), the donor *Lactobacillus plantarum* DG 507 (closed squares) and the trans-conjugants isolated for tetracycline resistance (open squares) and for erythromycin resistance (triangles) from faecal samples. The arrows indicate the time of donor administration. Each point represents the average of data from five rats, and error bars represent standard deviations.

The intestinal distribution of recipients, donors and TCs was relatively similar within the different rats, and also between those inoculated with different donor strains (Table 1). In all four intestinal segments, the recipient concentration was higher than the concentration of the donors, and these were in turn higher than the concentration of TCs (as also observed for the faecal samples). In all rats, bacterial concentrations were lower in the upper (duodenum and ileum) than in the lower (caecum and colon) intestinal segments (Table 1). In the duodenum, TCs were detected in only one of the rats (detection limit 2×10^1 CFU g^{-1} faeces). In the ileum, TCs were detected in half of the rats, whereas in caecum and colon TCs were observed in all but one rat. The number of Tet^r TCs reached an average of *c.* 2 log CFU g^{-1} in the two latter segments and slightly higher for the Erm^r TCs (Table 1).

Table 1. Intestinal distribution of recipients, donors and TCs from rats inoculated with (A) *Enterococcus faecalis* JH2-2 and *Lactobacillus plantarum* DG 522, and (B) *E. faecalis* JH2-2 and *L. plantarum* DG 507

	Duodenum	Ileum	Caecum	Colon
A				
E. faecalis JH2-2	6.20 (0.30)	7.08 (0.66)	8.50 (1.39)	9.31 (0.48)
L. plantarum DG 522	4.64 (0.69)	5.92 (0.81)	6.53 (1.51)	6.53 (0.34)
E. faecalis JH2-2,p*tet*(M)$_{DG\ 522}$	$1.30^{n=1}$	$1.86^{n=3}$ (0.31)	$2.49^{n=3}$ (0.33)	2.06 (0.60)
B				
E. faecalis JH2-2	5.62 (0.81)	7.01 (0.40)	8.88 (0.98)	9.33 (0.11)
L. plantarum DG 507	4.21 (1.49)	5.02 (1.11)	5.50 (0.77)	6.20 (0.34)
E. faecalis JH2-2,p*tet*(M)$_{DG\ 507}$	< LD	$2.11^{n=2}$ (1.15)	$1.95^{n=3}$ (0.87)	2.30 (0.82)
E. faecalis JH2-2,p*erm*(B)$_{DG\ 507}$	< LD	$2.30^{n=2}$ (0.99)	2.18 (0.94)	$3.28^{n=4}$ (0.50)

The numbers represent average log CFU g^{-1} segment of five rats. The standard deviations are given in parentheses. Several rats contained TCs at numbers below the limit of detection (LD) of 1.30 log CFU g^{-1} intestinal segment. In those cases where positive samples could not be obtained by all five rats, *n* indicates the number of samples used for calculation of the average value and standard deviation.

Verification of TCs by PCR

At least 25 isolates of each of the three types of TCs from the faecal samples were selected for verification. All the verified isolates were confirmed to be true TCs. They were demonstrated to be of the same species as the recipient as witnessed by a positive PCR reaction with primers specific for *E. faecalis*. In addition, it was confirmed by PCR that all tetracycline-resistant isolates [*E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$ and *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$] had received the *tet*(M) gene, and that all erythromycin-resistant isolates [*E. faecalis* JH2-2,p*erm*(B)$_{DG\ 507}$] had received the *erm*(B) gene (representative data are shown in Fig. 3). The recipient and donor strains were used as controls for the *E. faecalis*-specific PCR and for the resistance-gene-specific PCR. The controls gave the expected negative or positive results (Fig. 3).

Determination of cross-resistance

In order to determine whether the verified TCs *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$ and *E. faecalis* JH2-2,p*erm*(B)$_{DG\ 507}$ had received the *tet*(M) gene, the *erm*(B) gene or both genes from donor *L. plantarum* DG 507, parallel PCR reactions with the two gene-specific primer sets were performed. Among 54 examined isolates (27 of each TC type), one of each type had received both genes (data not shown), meaning that *c.* 4% of all TCs were cross-resistant to both tetracycline and erythromycin. This low ratio was supported by plating of faecal samples on BHI agar (supplemented with rifampicin, fusidic acid, tetracycline and erythromycin) selective for TCs with both resistance genes. These results showed that, in six positive duplicate samples taken from two rats during four individual days, the number of cross-resistant isolates accounted for *c.* 4% (±2% SD) and 17% (±7% SD) of the erythromycin- and the tetracycline-resistant isolates, respectively.

Determination of MIC values

The MIC of tetracycline and erythromycin was determined for the recipient, the donors and all the verified TCs. The MIC of tetracycline was 64–96 μg mL^{-1} in all examined tetracycline-resistant TCs. This value was lower than observed for the donors, which were resistant to > 256 μg mL^{-1}. The MIC for erythromycin was > 256 μg mL^{-1} in all the erythromycin-resistant TCs and similar to the value measured for the donor *L. plantarum* DG 507. The recipient *E. faecalis* JH2-2 was susceptible to both antibiotics and the MIC was < 1 μg mL^{-1}.

Plasmid profiles and Southern blotting

Among the verified TCs, ten isolates of each type (two from each of the five rats) were selected for further genotypic characterization of the transferred plasmids. In plasmid profiles of most of the *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$ isolates, the presence of a plasmid band corresponding to *c.* 40 kb was visible (Fig. 4A1). This band was not present in the plasmid-free recipient strain *E. faecalis* JH2-2 (data not shown), but matched the size of a band present in the plasmid profile of donor strain *L. plantarum* DG 522 (Fig. 4A1). Using Southern hybridization, the *tet*(M) probe showed a positive signal with this band in both the donor and the TCs (Fig. 4A2). In addition to the *c.* 40-kb band, hybridization to a second band with lower electrophoretic mobility was observed in the TC isolates and in the donor (Fig. 4A2). This band could represent a dimer of the same plasmid, but this possibility was not investigated further. In five of the *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$ isolates, two additional smaller bands were observed apart from the larger band corresponding to the resistance plasmid. These two bands appeared together in all cases, and hence two distinct plasmid profiles were observed for the examined isolates. Within most of the rats, both types of profiles were present (Fig. 4A1).

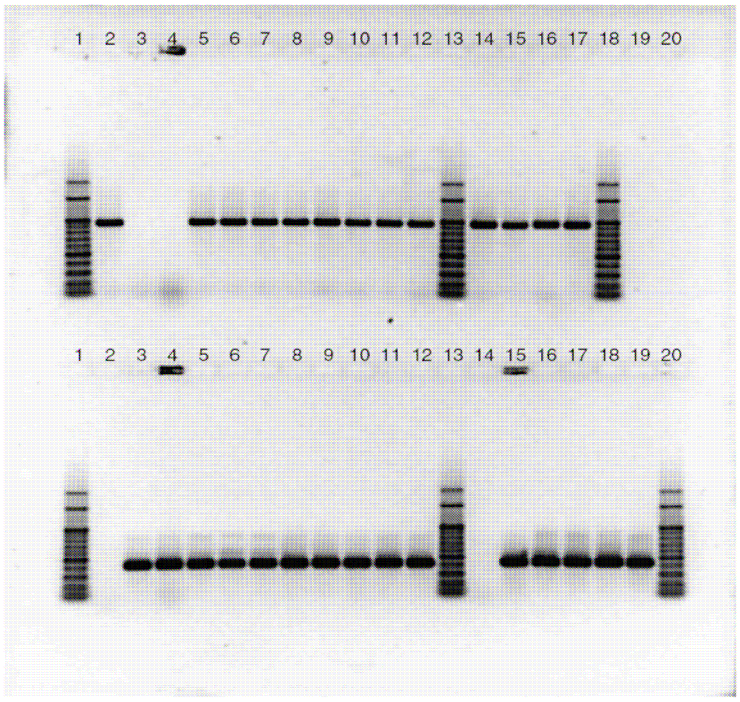

Fig. 3. PCR products of the three types of transconjugants (TCs), donors and recipient with primers specific for *Enterococcus faecalis* (lanes 2–12; 14–17), *tet*(M) (lanes 22–32) and *erm*(B) (lanes 34–39). For each TC type, four isolates taken from four different rats at different time points during the study are shown. Lane 2: *E. faecalis* JH2-2; lane 3: *Lactobacillus plantarum* DG 522; lane 4: *L. plantarum* DG 507; lanes 5–8: *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$ TC; lanes 9–12: *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$ TC; lanes 14–17: *E. faecalis* JH2-2,p*erm*(B)$_{DG\ 507}$ TC; lane 22: *E. faecalis* JH2-2; lane 23: *L. plantarum* DG 522; lane 24: *L. plantarum* DG 507; lanes 25–28: *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$ TC; lanes 29–32: *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$ TC; lane 34: *E. faecalis* JH2-2; lane 35: *L. plantarum* DG 507; lanes 36–39: *E. faecalis* JH2-2,p*erm*(B)$_{DG\ 507}$ TC; lanes 1; 13; 18; 21; 33; 40: 100-kb size marker (Viogene, Sunnyvale, CA; cat. MD101).

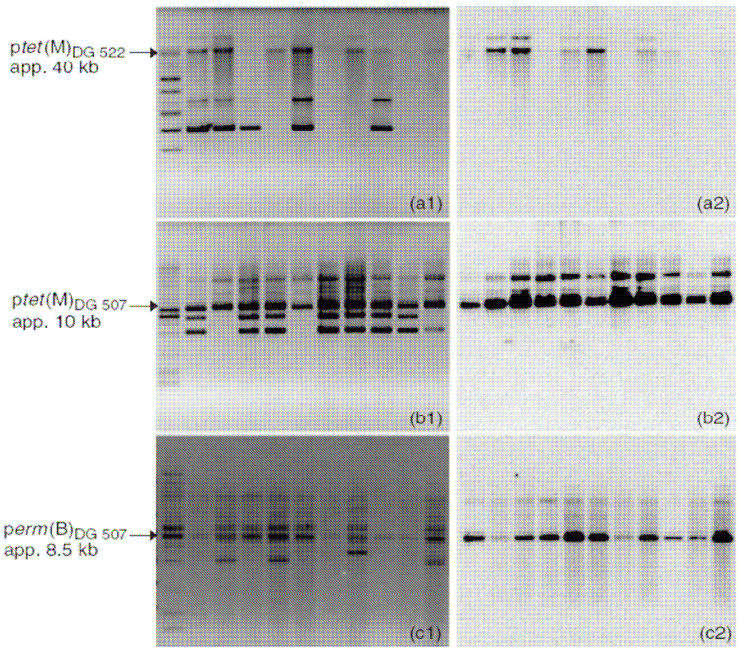

Fig. 4. Plasmid profiles (a1–c1, ethidium bromide-stained) of the donors and the three types of transconjugants (TCs) isolates and Southern blots and hybridization with the *tet*(M) (a2-b2) and *erm*(B) (c2) probes. (a1) Donor *Lactobacillus plantarum* DG 522 (lane 1) and 10 *Enterococcus faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$ TCs (lanes 2–11). (b1) Donor *L. plantarum* DG 507 (lane 1) and 10 *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$ TCs (lanes 2–11). (c1) Donor *L. plantarum* DG 507 (lane 1) and 10 *E. faecalis* JH2-2,p*erm*(B)$_{DG\ 507}$ TCs (lanes 2–11). The TCs are run pairwise, two from each of the five rats. a2–c2 show the corresponding Southern blots for a1–c1, respectively.

Characterization of the plasmid profiles was also performed for 10 tetracycline- and 10 erythromycin-resistant isolates selected from mating pair DG 507/JH2-2. In the *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 507}$ isolates, a bright plasmid band of *c.* 10 kb was evident in all isolates as well in the donor *L. plantarum* DG 507 (Fig. 4B1). Likewise, in the *E. faecalis* JH2-2,p*erm*(B)$_{DG\ 507}$ TCs a band of *c.* 8.5 kb was evident in all isolates, which corresponded to a similar size band in the donor (Fig. 4C1). These two bands showed positive hybridization with probes for *tet*(M) and *erm*(B), respectively (Fig. 4B2–C2). Again, hybridization to more than one band was observed with both the *tet*(M) and the *erm*(B) probe, possibly owing to different forms of the plasmids. The bands that hybridized with the probes were the same in both the donor and all the TCs. As for *E. faecalis* JH2-2,p*tet*(M)$_{DG\ 522}$ TCs, co-transfer of plasmids other than the resistance plasmids was detected in several of the TC isolates derived from donor *L. plantarum* DG 507, and different plasmid profiles appeared within the same rat (Fig. 4B1–C1).

In vitro mating

In vitro transferability of the *tet*(M) and the *erm*(B) resistance genes from 15 distinct TC isolates was investigated using a filter mating procedure. No transfer was observed to recipients *E. faecalis* JH2SS and *E. faecalis* OG1SS (the detection limit was $< 10^{-8}$ TCs/recipient). The positive control (transfer of pAMβ1 from *E. faecalis* JH2-2 to *E. faecalis* JH2SS) was performed simultaneously with mating between the TCs and *E. faecalis* JH2SS. The control showed high transfer rates of 1.7×10^{-3} TCs/recipient.

Discussion

To our knowledge, this is the first study to demonstrate *in vivo* transfer of wild-type antibiotic resistance plasmids from *L. plantarum* to *E. faecalis*. Only a limited number of studies have investigated antibiotic resistance transfer from *Lactobacillus* and these studies have concentrated on the introduced broad-host-range conjugative plasmid pAMβ1 encoding resistance to macrolide lincosamide and streptogramin B antibiotics. As such, it has been shown that *L. plantarum, L. reuteri, L. fermentum* and *L. murinus* can function as donors of pAMβ1 to other lactic acid bacteria *in vitro* (West & Warner, 1985; Shrago *et al.*, 1986; Tannock, 1987) and in the gastrointestinal tract of mice (Morelli *et al.*, 1988; McConnell *et al.*, 1991). Likewise, it has been shown that a mobile native plasmid encoding chloramphenicol resistance can be transferred from *L. plantarum* to *Carnobacterium piscicola* by means of co-mobilization with pAMβ1 (Ahn *et al.*, 1992).

The implementation of this study was brought about by the finding that wild-type *L. plantarum* strains isolated from Belgian fermented dry sausages were able to transfer tetracycline and erythromycin resistance genes to *E. faecalis* JH2-2 in filter mating experiments (Gevers *et al.*, 2003b). However, the gastrointestinal tract is a very hostile environment to allochthonous bacteria, and *in vitro* transfer of plasmids is therefore not necessarily synonymous with transfer *in vivo*. A very important barrier that the ingested bacteria meet in passage through the gastrointestinal tract is the low pH and bile as encountered in the stomach and the upper intestine. Earlier studies have shown that strains of *L. plantarum* can be rather resistant to these conditions, and thus have a relatively good survival rate during transit of the gastrointestinal tract (Johansson *et al.*, 1998; Vesa *et al.*, 2000; Bron *et al.*, 2004; Goossens *et al.*, 2005). In contrast, it has been shown that several food-associated *Lactobacillus* species, such as *L. curvatus* and *L. Sakei*, were detectable in faeces only by molecular methods and not by culturing, perhaps because they were dead or in a non-culturable state (Walter *et al.*, 2001).

In the present study, the two donor strains were introduced to the rats through multiple doses, thus simulating a worst-case scenario of daily intake of food products containing the resistant bacteria. The intestinal establishment of the donors was not investigated in this study, but the colonization potential would probably be different from that in a normal intestine, where the indigenous microbiota would represent a much greater colonization resistance. However, whereas the physiological state of the donor bacteria is critical for conjugation of resistance genes, the ability to colonize is not a prerequisite. Hence, several studies have shown transfer from donors, that were quickly eliminated from the intestine (Gruzza *et al.*, 1993; Gruzza *et al.*, 1994; Schlundt *et al.*, 1994; Walter *et al.*, 2001; Licht *et al.*, 2002).

In this study, TCs were generated exhibiting resistance to either tetracycline or erythromycin or both. The TCs made up *c.* 10^{-7} of the recipient population at the end of the experiment, which is a low fraction taking the *in vitro* transfer rates of 10^{-4}–10^{-5} TCs/recipient into consideration. However, one should keep in mind that the gastrointestinal tract is a dynamic system and that mating *in vivo* is a very complex scenario, both in time and in space, compared with filter mating. Consequently, a direct comparison with *in vitro* transfer ratios is not feasible. In the *in vivo* situation, three events may have participated in the development of TCs: (1) primary transfer of resistance genes from the *Lactobacillus* donors to the recipients; (2) secondary transfer from TCs to recipients; and (3) growth of TCs. Clearly, the contribution of each of these events is difficult to assess and was outside the scope of this study. However, it is obvious that primary transfer of resistance genes from the *Lactobacillus* donors is the prerequisite for establishing a TC population. The ability of the TCs to facilitate secondary transfer of the resistance plasmids was evaluated by *in vitro*

matings, but no such transfer could be detected. However, these results do not rule out the possibility that the TCs can indeed function as donors under different conditions from the one tested here.

It was thought that the sub-therapeutic administration of antibiotics to the rats in this study would promote the spread of antibiotic resistance genes, because this effect had been reported previously (Salyers & Shoemaker, 1996; Licht *et al.*, 2003). However, this effect was not observed. The population size of the sensitive recipient strain *E. faecalis* JH2-2 did not decrease in response to the antibiotics, but the expected increase in donors and TCs was not evident either. Possibly, the sub-therapeutic concentration of tetracycline was too low. This assumption is supported by the findings of Bahl *et al.* (2004), who showed that the concentration of bioavailable tetracycline within the bacterial growth habitat of the intestine represented only *c.* 0.4% of the intake concentration of the antibiotic.

Ultimately, the true risk of antibiotic resistance plasmids circulating in food and in the intestinal environment is the eventual transfer of resistance to human pathogens. The findings of our study show that *in vivo* resistance gene transfer can take place from wild-type *L. plantarum* isolated from food to *E. faecalis*, which represent a natural inhabitant of the human gut and a potentially pathogenic species. However, as the present experiment was performed in conditions with artificially high numbers of recipients and in the absence of competition, the extent of this potential safety hazard should be further investigated. For instance, animal models with a conventional biota or human-biota-associated animals could be used to investigate the magnitude of resistance gene transfer in the presence of a colonization barrier or during competition from a bacterial population imitating that of a human. The number of transfer events in more complex models will most likely be very restricted compared with that in the gnotobiotic gut. On the other hand, the persistence and growth advantage of indigenous recipients that receive a resistance gene under antibiotic selective conditions may be much improved. Hence, the maintenance and potential growth of TC populations during antibiotic treatment is an additional issue that needs to be addressed in future studies.

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Paper II

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Selective pressure affects transfer and establishment of a *Lactobacillus plantarum* resistance plasmid in the gastrointestinal environment

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Objectives and methods: A *Lactobacillus plantarum* strain recently isolated from French raw-milk cheese was tested for its ability to transfer a small plasmid pLFE1 harbouring the erythromycin resistance gene *erm*(B) to *Enterococcus faecalis*. Mating was studied *in vitro* and in different gastrointestinal environments using gnotobiotic rats as a simple *in vivo* model and streptomycin-treated mice as a more complex model. Transfer and establishment of transconjugants in the intestine were investigated with and without selective pressure.

Results: Compared with the relatively low transfer frequency of $\sim 5.7 \times 10^{-8}$ transconjugants/recipient obtained *in vitro* by filter mating, a surprisingly high number of transconjugants (10^{-4} transconjugants/recipient) was observed in gnotobiotic rats even without antibiotic treatment. When erythromycin was administered, a transfer rate of ~100% was observed, i.e. the recipient population turned completely into transconjugants (3×10^9 cfu/g faeces). Additionally, the time to reach a stable transconjugant population level was much faster in the erythromycin-treated gnotobiotic rats (1 day) than in the untreated animals (4–5 days). Transconjugants persisted in the gut in relatively stable numbers at least 12 days after termination of antibiotic treatment. In the streptomycin-treated mice, no transfer was observed either with or without erythromycin treatment.

Conclusions: The overall results imply that the gastrointestinal tract may comprise a more favourable environment for antibiotic resistance transfer than conditions provided *in vitro*. However, the indigenous gut microbiota severely restricts transfer, thus minimizing the number of detectable transfer events. Treatment with erythromycin strongly favoured transfer and establishment of pLFE1.

Keywords: *L. plantarum*, antibiotic resistance, horizontal gene transfer, gastrointestinal tract

Introduction

During recent years, the question of whether lactobacilli can function as a pool for antibiotic resistance genes has gained much attention.[1,2] Lactobacilli are industrially valuable bacteria, which are augmented in large viable numbers both in probiotic products and as starter cultures in a range of fermented milk and meat products. Lactobacilli also occur frequently in food produced from undefined starter cultures and as unintended contaminations. Many studies have selected and identified antibiotic-resistant species of lactobacilli from various environments encompassing different parts of the food chain, e.g. probiotics and starter cultures,[3,4] fermented milk products,[5–7] cheese,[8,9] meat products[10,11] and human isolates.[12] When associated with mobile genetic elements such as plasmids or transposons, antibiotic resistance determinants can potentially be transferred to the commensal intestinal microbiota or pathogens transiently present in humans. Literature on conjugative transfer of naturally occurring antibiotic-resistance determinants in lactobacilli is nevertheless rare. Previously, transfer of native

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tetracycline and erythromycin resistance plasmids from two food-associated *Lactobacillus plantarum* strains to *Enterococcus faecalis* was observed in gnotobiotic rats.[13] Gevers *et al.*[14] showed *in vitro* conjugation from different species of lactobacilli to *E. faecalis* and *Lactococcus lactis* recipients but not to a *Staphylococcus aureus* recipient. In contrast, two other studies found no indications of intraspecies (*Lactobacillus* donor–recipient combinations) or interspecies (from lactobacilli to *E. faecalis*, *Enterococcus faecium* or *L. lactis*) resistance transfer.[3,4] However, extrapolation of *in vitro* data to naturally occurring environments in the gut is not straightforward due to the limited knowledge of transfer mechanisms and factors affecting transferability within the gastrointestinal tract.

In this study, we examined the transfer of a native erythromycin resistance plasmid (pLFE1) from a *L. plantarum* strain isolated from a French raw-milk cheese to *E. faecalis*, a common inhabitant of the normal human gut microbiota, and also an opportunistic pathogen. Mating was investigated in two *in vivo* models with no or decreased colonization resistance represented by germ-free rats and streptomycin-treated mice, respectively, and *in vitro* by filter-mating. Additionally, the effect of different concentrations of erythromycin on the transfer rate and the persistence of pLFE1-carrying transconjugants in the gastrointestinal tract was investigated.

Materials and methods

Bacterial strains

The donor strain used in this study was *L. plantarum* M345 isolated in 2005 from a French raw-milk cheese (Tomme de Savoie) according to IDF standard 122C:1996.[15] *L. plantarum* M345 harbours a small mobile plasmid (pLFE1) of ~4 kb, which, among others, contains an *erm*(B) erythromycin resistance gene. For the *in vivo* experiment with streptomycin-treated mice, a spontaneously streptomycin-resistant mutant M345S of M345 was isolated and used as the donor. Before the experiment was initiated, the plasmid profile of both variants and *in vitro* transfer rate of pLFE1 was checked and no differences were observed between the streptomycin-resistant mutant and the wild-type strain. *E. faecalis* JH2SS showing resistance to streptomycin and spectinomycin ($Strep^r$, $Spec^r$)[16] was used as recipient strain. *E. faecalis* JH2-2[17] isogenic to *E. faecalis* JH2SS and resistant to rifampicin and fusidic acid was used as a recipient to test for secondary transfer of pLFE1 from transconjugants of *E. faecalis* JH2SS *in vitro*.

Culture conditions

In vivo experiment with gnotobiotic rats and in vitro analyses. For all *in vitro* analyses and preparation of inoculation cultures for the gnotobiotic rat experiment, the donor strain was grown anaerobically at 37°C for 24–48 h in de Man, Rogosa and Sharpe (MRS) medium (Oxoid, Hampshire, UK) supplemented with 50 mg/L erythromycin (Sigma, Bornem, Belgium). For determination of donor cfu from the rat samples, Rogosa agar (Oxoid) supplemented with 50 mg/L erythromycin was used. The recipient was grown aerobically at 37°C for 24 h in brain heart infusion (BHI) (Oxoid) supplemented with 500 mg/L streptomycin and 500 mg/L spectinomycin (Sigma) for the *in vitro* analyses and inoculation culture for the gnotobiotic rat experiment. Selection of the recipient from the rat samples was performed on Slanetz and Bartley agar (Oxoid) supplemented with the appropriate antibiotics and incubated at 42°C for 48 h. Transconjugants were selected from the rat samples on Slanetz and Bartley agar (Oxoid) supplemented with streptomycin and spectinomycin (both at 500 mg/L) and 16 mg/L erythromycin and incubated under aerobic conditions at 42°C for 72 h [limit of detection (l.d.) = 20 cfu/g faeces]. In subsequent analyses, transconjugants were grown in BHI media at 37°C for 24–48 h.

In vivo experiment with streptomycin-treated mice. The donor strain was selected from mice faecal samples on MRS agar plates supplemented with 100 mg/L streptomycin and 50 mg/L erythromycin. The recipient was selected on Enterococcosel agar (BD Diagnostics) supplemented with 500 mg/L streptomycin and 500 mg/L spectinomycin. Plates selective for transconjugants were Enterococcosel agar containing 500 mg/L streptomycin, 500 mg/L spectinomycin and 50 mg/L erythromycin (l.d. = 20 cfu/g faeces), and Enterococcosel agar containing 500 mg/L spectinomycin and 50 mg/L erythromycin (l.d. = 10 cfu/g faeces). The plates were incubated at 37°C for 48 h (donor and recipient) or 72 h (transconjugants). The donor was incubated under anaerobic conditions and the recipient and transconjugants were incubated under aerobic conditions.

Animal management and in vivo set-up

The animal experiments were approved and conducted according to Danish national legislation.

Gnotobiotic rats. Fifteen germ-free Sprague–Dawley rats (13 males and 2 females) were bred at the National Food Institute from parents originally supplied by Taconic (Germantown, NY, USA). The rats were ~6 weeks old at the beginning of the experiment. Housing and feeding were as previously described,[18] except the rats were caged two or three (belonging to the same group) together and walking on gratings. The rats were placed in three groups of five animals: group A, a control group (including the two female rats), which did not receive erythromycin; group B, a group treated with a low concentration of erythromycin; and group C, a group treated with a higher concentration of erythromycin. The high concentration of erythromycin used in the present study was based on the recommended clinical dose for children, which is calculated on the basis of body weight. This concentration was then scaled down to the size of the rats and mice. Furthermore, in order to mimic the intestinal absorption ways as best as possible, a therapeutic preparation of erythromycin (ABBOTICIN, Abbott Laboratories Ltd, Queensborough, UK) was used, which contains erythromycin in the form of erythromycinethylsuccinate (for concentrations, see below).

At day 0, all rats received 4×10^8 cfu of the recipient strain *E. faecalis* JH2SS per os by gavage. Six days after introduction of the recipient, the donor strain was introduced. During five consecutive days, bacteria from a fresh overnight culture of the donor strain were harvested and resuspended in phosphate-buffered saline (PBS) (Oxoid) and offered instead of drinking water. The rats were allowed free access to the solution, which contained ~3×10^{10} cfu/mL donor bacteria.

During the period of donor dosing, the rats in groups B and C received two daily doses each of 0.25 mg/animal and 2.5 mg/animal erythromycin, respectively. The erythromycin was given per os by gavage. Fourteen days after the end of erythromycin treatment and donor dosing, all rats were euthanized at day 24.

Streptomycin-treated mice. Twenty-four outbred albino female NMRi and 24 inbred BALB/c mice aged 6–8 weeks were supplied from Taconic (Ejby, Denmark). The mice were caged in pairs and the cages were changed daily. From day '−1', the mice continuously received drinking water containing 1 mg/mL streptomycin sulphate

(Sigma, St Louis, MO, USA). The animals from each breed were placed in four groups of six mice: group A, only received the recipient; group B, received the recipient and erythromycin treatment; group C, received the recipient and the donor; and group D, received the recipient, the donor and erythromycin treatment.

The selectivity of the applied agar plates for donor, recipient and transconjugants cultivation from faecal samples was confirmed prior to recipient inoculation (day −1). Owing to sporadic growth of faecal bacteria on the recipient plates, two mice were excluded from the experiment. The recipient (10^8 cfu) was inoculated to all mice at day 0 and re-inoculated again at day 10. Starting at day 7, mice belonging to groups C and D were inoculated daily with $\sim 5.0 \times 10^8$ cfu of the donor strain. During the period of donor dosing, mice in groups B and D were given one daily dose of 0.025 mg erythromycin (ABBOTICIN). All bacterial inoculums were overnight cultures washed and resuspended in 20% (w/v) sucrose (Sigma) and all inoculations were done per os. On day 17, all mice were euthanized.

Collection and treatment of in vivo samples

Gnotobiotic rats. To verify the germ-free status of the rats, faecal samples were collected and analysed for aerobic and anaerobic growth of bacteria and yeasts before the experiment was initiated. Faecal samples were collected directly from the rats by careful squeezing of their abdomen. At euthanization, samples from duodenum, ileum, caecum and colon were taken. All samples were homogenized by whirly mixing in PBS. Ten-fold dilution series were prepared in PBS and incubated on the appropriate selective agar-plates for enumeration of donors, recipients and transconjugants as described above.

Streptomycin-treated mice. Faecal samples (0.5 g/cage, originating from two animals) shed within 24 h were collected. Numbers of cfu of donors, recipients and transconjugants were determined on the selective plates as described above.

Verification of transconjugants by PCR

Selection of transconjugants was based on their phenotypic resistance profile. In order to confirm that these isolates were in fact true transconjugants and not mutants, PCR assays with primers species-specific for *E. faecalis* and targeting the *erm*(B) resistance gene were carried out as previously described.[13] Altogether, 45 transconjugant isolates from different animals, groups and days during the study were tested. The donor and recipient strain were included as positive and negative controls.

Determination of MIC values

The MIC of erythromycin was determined for nine transconjugants isolated from the animals as well as for *L. plantarum* M345 and *E. faecalis* JH2SS. Overnight cultures were prepared in broth according to standard conditions. The cultures were streaked onto Mueller–Hinton (BBL, Sparks, USA) agar plates for transconjugants and the recipient and on MRS agar plate for the donor, respectively. Etest strips (AB Biodisk, Solna, Sweden) were applied and the plates were read after overnight incubation at appropriate conditions for donor, recipient and transconjugants.

Plasmid profiles and Southern blotting

Plasmid DNA was isolated from nine transconjugants (representing nine rats and three groups) and the donor and recipient strain. We used the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, except that an initial lysozyme step (2 mg/mL lysozyme in P1 buffer for 25 min at 37°C) was included. The plasmid extractions were cut with *Xcm*I (New England Biolabs), which cuts pLFE1 uniquely outside the *erm*(B) probe (sequencing results, unpublished results) and both undigested and digested profiles were run on a 0.7% agarose gel. The gel was blotted and hybridized with an *erm*(B) probe as previously described.[13]

Secondary transfer of erm(B) plasmid pLFE1 from transconjugants

The ability of *E. faecalis* JH2SS transconjugants obtained from the animals to function as new donors of the *erm*(B) gene was tested by a filter mating assay. Twenty transconjugant isolates verified by PCR were assessed as donors and *E. faecalis* JH2-2 (Rif^r, Fus^r) was used as an isogenic recipient. The strains were grown in BHI broth supplemented with the respective antibiotics to an $OD_{600} = 0.7–1.0$. Four donor mixtures were then prepared from the 20 transconjugants by mixing each of five transconjugant cultures together in equal volumes. Thereafter, donor mixture and recipient culture were mixed in equal volumes and vacuum-filtered onto sterile filters (HAWP04 700, Millipore, Bedford, MA, USA), which were incubated under aerobic conditions on non-selective BHI agar plates at 37°C overnight. The bacteria were washed off the filters with PBS and appropriate dilutions spread onto plates selective for the donor (BHI + 500 mg/L streptomycin, 500 mg/L spectinomycin and 16 mg/L erythromycin), recipient (BHI + 50 mg/L rifampicin and 25 mg/L fusidic acid) and transconjugants (BHI+16 mg/L erythromycin, 50 mg/L rifampicin and 25 mg/L fusidic acid). As control, the donor mixture and the recipient culture were each mixed with PBS and placed separately on filters.

Effect of erythromycin on transfer frequency in vitro

The effect of erythromycin concentration on *erm*(B) plasmid transfer frequency from *L. plantarum* M345 to *E. faecalis* JH2SS *in vitro* was tested. Four different concentrations 0.05, 0.5, 1 and 5 mg/L erythromycin were added to the BHI mating plates. A control without erythromycin was also included. The donor and recipient strains were grown in broth supplemented with the respective antibiotics to $OD_{600} = 0.9–1.1$ The bacteria were mixed in equal volumes and vacuum filtered onto sterile filters (HAWP04 700), which were placed on the mating plates containing the various concentrations of erythromycin. The plates were incubated under aerobic conditions at 37°C overnight and the bacteria subsequently washed off and prepared on plates selective for the donor, recipient and transconjugants. Controls of separate donor and recipient plates were also prepared. The experiment was performed in five replicates.

Statistics

The effect of erythromycin treatment on the number of recipients and transconjugants in the *in vivo* experiments was calculated using the analysis of variance with interaction. The number of recipients or transconjugants was set as the response variable, and for the gnotobiotic rat experiment, rats were specified as random effect, and

group and time as fixed effects. A similar analysis was made for the streptomycin-treated mice, where cages were specified as random effect and mouse-breed, time and donor inoculation as fixed effects. For pair-wise testing, Student's *t*-test was used.

Results

Conjugal transfer in gnotobiotic rats

Transferability of a wild-type *erm*(B) plasmid from *L. plantarum* M345 to *E. faecalis* JH2SS was studied in gnotobiotic rats. Strain *E. faecalis* JH2SS was given as a single dose to all rats at day 0. The strain readily colonized the rats at levels of $\sim 5 \times 10^9$ cfu/g faeces (Figure 1). At days 6–10, the donor strain was introduced via the drinking water. During the same period, rats belonging to groups B and C received erythromycin in medium and high concentration, respectively, whereas rats belonging to group A received no antibiotic treatment. Erythromycin had a major effect on the rate of establishment as well as on the size of transconjugant populations obtained. Two days after introduction of the donor strain, transconjugants were detected in rats from all groups (Figure 1). When erythromycin was administered, the increase in numbers of transconjugants occurred much faster (1 day) than that observed in animals without antibiotic treatment (4–5 days), after which a stable population level was reached. Furthermore, the size of the transconjugant population, which was established in the rats, was approximately 4 log units higher in groups B and C ($\sim 3 \times 10^9$ cfu/g faeces) than in group A ($\sim 5 \times 10^5$ cfu/g faeces), the former being an almost complete turnover of recipients into transconjugants (Figure 1).

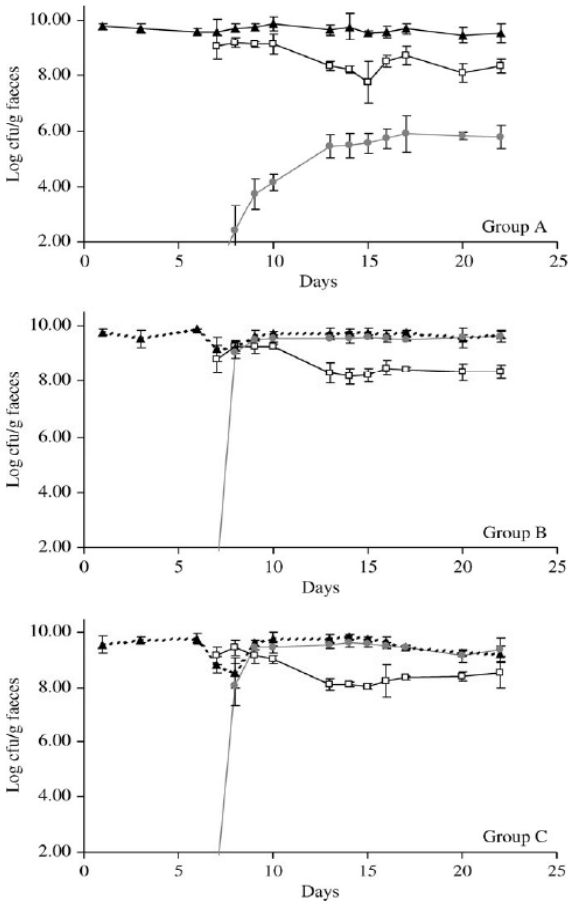

Figure 1. cfu counts of donors (squares), recipients+transconjugants (triangles) and transconjugants (circles) from faecal samples of rats without antibiotic treatment (group A), with 0.25 mg of erythromycin twice a day (group B) and with 2.5 mg of erythromycin twice a day (group C). Each point represents the average from five rats and error bars indicate SDs. The donor strain and antibiotics were added to the rats at days 6–10.

Not surprisingly, erythromycin affected the recipients. After initiation of erythromycin treatment, the number of recipients decreased significantly both in groups B ($P < 0.01$) and C ($P = 0.001$). A significantly larger decrease ($P < 0.05$) in the number of recipients was observed in the rats treated with a high concentration of erythromycin than in rats treated with a medium concentration of the antibiotic. However, after establishment, no significant ($P = 0.73$) differences in transconjugant numbers could be seen between rats treated with high and medium concentrations of erythromycin (Figure 1). Erythromycin did not seem to have any effect on colonization of the donor strain. Thus, the donor was retrieved from rat faeces in numbers of $\sim 10^9$ cfu/g faeces, irrespective of erythromycin treatment (Figure 1). After discontinuation of donor and erythromycin dosing at day 10, the number of donors decreased to $\sim 2 \times 10^8$ cfu/g faeces in all rats and remained relatively constant throughout the rest of the study.

Cultivation of bacteria from intestinal segments

The numbers of donors, recipients and transconjugants were higher in the upper end of the intestine (duodenum and ileum) than in the lower intestinal segments (caecum and colon) (Table 1). Transconjugants were present in all segments indicating that transfer occurred relatively soon in the gastrointestinal tract. The ratio between donors, recipients and transconjugants was approximately the same in all segments.

Verification of transconjugants

PCR was carried out using primers specific for the *erm*(B) gene and for *E. faecalis* to verify the transconjugants that were selected during the *in vivo* experiments solely by their resistance phenotype. All 45 transconjugants subjected to PCR analysis gave positive products both with the *erm*(B) and the *E. faecalis* specific primers (data not shown). As expected, *L. plantarum* M345 and *E. faecalis* JH2SS gave positive and negative reactions, respectively, with the *erm*(B) PCR primers. The opposite result was obtained with *E. faecalis* specific primers, confirming the reliability of the analysis. MIC of erythromycin was determined for *L. plantarum* M345, *E. faecalis* JH2SS and nine transconjugants from the rats. All transconjugants were resistant to erythromycin with an MIC >256 mg/L. The recipient strain was sensitive (MIC 0.25 mg/L) and the donor was resistant (MIC > 256 mg/L).

Table 1. Distribution of donors, recipients+transconjugants and transconjugants in intestinal segments of rats belonging to group A (control), group B (medium erythromycin concentration) and group C (high erythromycin concentration)

Isolate	Group	Duodenum	Ileum	Caecum	Colon
L. plantarum M345	A	1.5×10^5	5.5×10^6	1.0×10^7	8.3×10^7
	B	7.4×10^5	4.9×10^6	5.4×10^6	9.1×10^7
	C	2.0×10^5	3.9×10^5	5.4×10^6	7.9×10^7
E. faecalis JH2SS+transconjugants	A	1.0×10^6	1.5×10^8	3.5×10^8	1.6×10^9
	B	4.4×10^6	3.5×10^8	1.7×10^8	1.6×10^9
	C	8.9×10^5	3.0×10^7	4.2×10^8	8.9×10^8
Transconjugants	A	1.6×10^{2a}	5.2×10^4	5.1×10^4	2.8×10^5
	B	3.6×10^6	2.0×10^8	1.4×10^8	1.5×10^9
	C	6.6×10^5	2.3×10^7	3.4×10^8	1.3×10^9

The numbers represent the geometric average cfu/g segment of five rats.
[a]The number is based on calculation from only two rats, since the other three were below the l.d. (40 cfu/g segment).

Plasmid profiles and Southern blotting

Plasmids were extracted from the donor *L. plantarum* M345, the recipient *E. faecalis* JH2SS and nine transconjugant isolates isolated from the gnotobiotic rat samples. The plasmid profiles were digested with *Xcm*I, blotted and hybridized with an *erm*(B)-specific probe. In the recipient strain, no plasmids were identified. In contrast, the donor and all transconjugants contained a plasmid band of ~4 kb, which hybridized with the *erm*(B) probe (Figure 2). In the digested profiles, the 4 kb band appears sharp and distinct, whereas the undigested profiles show a larger smear. This smear is presumably due to various forms of the plasmid migrating with different velocities during electrophoresis.

A second band of ~5 kb, which the *erm*(B)-specific probe did not hybridize with, was clearly present in the transconjugant-digested profiles. As the donor harbours several other plasmids in addition to pLFE1 (Figure 2), we believe that the ~5 kb band originates from another co-transferred plasmid.

Secondary transfer of erm(B) plasmid in vitro

The potential of transconjugants obtained *in vivo* to transfer the *erm*(B) plasmid to new recipients was investigated by filter mating. However, under the experimental conditions applied (detection limit was 10^{-9} transconjugants/recipient), no transfer was detected to the recipient *E. faecalis* JH2-2 (data not shown).

Effect of erythromycin on in vitro transfer frequency

The effect of erythromycin on selection of transconjugants was investigated *in vitro* by application of different concentrations of erythromycin to the agar plates used during mating. The number of transconjugants increased significantly when low concentrations of erythromycin were added (Table 2). The highest transfer frequency of 2.0×10^{-6} transconjugants/recipient was observed at 0.50 mg/L erythromycin. At higher erythromycin concentrations, the frequency decreased again to levels comparable to the control. The recipient was significantly ($P < 0.05$) inhibited by $\geq$0.50 mg/L erythromycin, as also expected from the MIC value of 0.25 mg/L. In contrast, the donor strain was unaffected by erythromycin.

Conjugation study in streptomycin-treated mice

Two different breeds of mice NMRi and BALB/c were used to study conjugal transfer in a gastrointestinal environment with a complex microbiota, where the colonization barrier was reduced by streptomycin treatment. Similar colonization levels of the donor and recipient strain were observed for the two breeds (data not shown). The recipient was introduced in all mice at

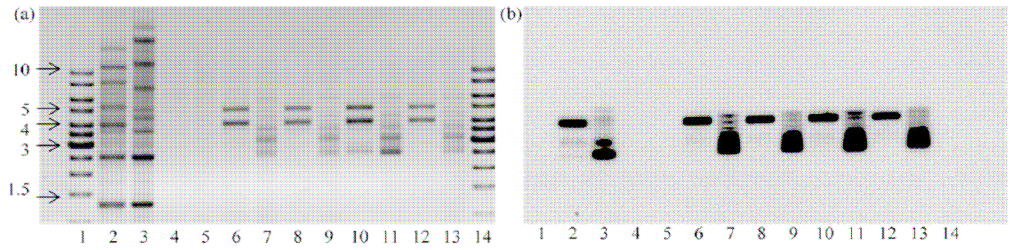

Figure 2. Plasmid profiles undigested or cut with restriction enzyme *Xcm*I. (a) Ethidium bromide staining of (b) the corresponding Southern blot hybridized with the *erm*(B) probe. Lanes 2 and 3, *Lactobacillus plantarum* strain M345; lanes 4 and 5, *Enterococcus faecalis* JH2SS; lanes 6–13, four different transconjugants representing different rats and groups. Each isolate is shown first digested and then undigested in lanes next to each other. Lanes 1 and 14, 1 kb GeneRuler (Fermentas). The numbers to the left of (a) are the sizes in kb of the GeneRuler linear DNA marker.

Table 2. Effect of different concentrations of erythromycin in the mating medium on *in vitro* transfer frequency between *L. plantarum* M345 and *E. faecalis* JH2SS

Erythromycin (mg/L)	Donor	Recipient+transconjugants	Transconjugants	Transconjugants/recipient
0.00	5.4×10^8	1.0×10^9a	5.6×10^1a	5.7×10^{-8} (1.54×10^{-8})a
0.05	5.8×10^8	9.1×10^8a	6.0×10^1a	1.1×10^{-7} (1.0×10^{-7})a
0.50	5.8×10^8	3.4×10^8b	5.2×10^2b	2.0×10^{-6} (1.4×10^{-6})b
1.00	5.9×10^8	3.4×10^8b	2.9×10^2b	9.1×10^{-7} (4.2×10^{-7})b
5.00	5.8×10^8	2.3×10^8c	1.9×10^1c	9.6×10^{-8} (5.7×10^{-8})a

The numbers are geometric average cfu/mL filter wash-off from five replicates.
SDs of the transfer frequencies are given in parentheses.
Different letters in a column indicate statistical significant differences in the concentrations or transfer frequencies obtained.

day 0 and colonized at least transiently at relatively high levels. At day 1, $\sim 10^9$ cfu/g faeces were recovered from faeces. During the next 1–2 days, the cfu numbers decreased $\sim$1 log unit. For the remaining part of the study, the population number was constant to slightly decreasing; thus at the end of the experiment (day 17), $\sim 10^7$ cfu/g faeces of the recipient was recovered from mice not treated with erythromycin (groups A and C, data not shown). In contrast, treatment with erythromycin (groups B and D) from day 7 resulted in a significant ($P < 0.001$) decrease in the recipient of $\sim$2–3 log units. Despite an increase after re-inoculation with the recipient at day 10, the numbers decreased to $\sim 10^5$ cfu/g faeces at day 17 in the erythromycin-treated groups (data not shown). The donor was introduced in the mice (groups C and D) at day 7 and was repeatedly inoculated each day throughout the experiment. As expected, erythromycin treatment did not affect the donor colonization, which remained relatively constant between 10^6 and 10^7 cfu/g faeces in all mice. No transconjugants were detected in this study (l.d. = 10 cfu/g faeces) (data not shown).

Discussion

In the present study, mobilization of a small, naturally occurring erythromycin resistance plasmid from *L. plantarum* was studied *in vitro*, as well as in two different gastrointestinal *in vivo* models. Gnotobiotic rats were used as a high transfer model to evaluate the transfer potential of the *L. plantarum* donor strain in a gastrointestinal environment without a colonization barrier. In this model, a high concentration of transconjugants was observed even when no selective pressure was applied (Figure 1, group A). Interestingly, the ratio of transconjugants to recipients was 3–4 log units higher in the gnotobiotic model than *in vitro*. This result is in agreement with a recent study of Dahl *et al.*,[19] investigating conjugation between different strains of *E. faecium*. In 9 out of 12 mating pairs, they observed higher ratios of transconjugants to recipients in gnotobiotic mice than in a filter-mating assay. They therefore suggested that transfer rates determined *in vitro* underestimate the transfer potential in the mammalian gastrointestinal tract. However, in a recent study, the opposite relationship between *in vitro* and *in vivo* transconjugants to recipient ratios was observed. *In vitro* transfer frequencies of plasmids harbouring *tet*(M) and *erm*(B) resistance genes from two strains of *L. plantarum* to *E. faecalis* were 3–4 log units higher than the ratio observed in gnotobiotic rats.[13] A possible explanation for these differences could be the colonization ability of the donor strain, which in the current study reached a population level of 10^8–10^9 cfu/g faeces, whereas in the earlier study, only 10^6–10^7 cfu/g faeces of the donor strains were observed.[13] A high donor density will in theory increase the encounters of donors and recipients, and thus increase mating events.[20] However, a vast number of other factors have also been proposed to affect the development of a specific plasmid-carrying population in the gut; for instance, the donor potential or the genetic advantages/disadvantages of the specific plasmid to the recipient population.[21] Thus, the efficiency of plasmid dissemination in the gastrointestinal tract cannot be predicted from extrapolation of *in vitro* mating experiments but requires more complex environments.

Erythromycin treatment had a very pronounced effect on dissemination of the *erm*(B) resistance plasmid pLFE1 in the gnotobiotic gut (Figure 1, groups B and C). We hypothesize that the increase in plasmid-carrying recipients was not only the result of a growth advantage during selective pressure, but also a direct effect on the number of transfer events. The transconjugants found in the *in vivo* samples could have evolved both horizontally from transfer and vertically by growth of the transconjugants. However, the relative contribution from each of these factors cannot be deduced from the present experimental set-up. Nevertheless, there is an upper limit to the rate with which the bacteria can grow and thus to the maximum contribution from the vertical factor. In the erythromycin-treated gnotobiotic rats, the initial increase in transconjugant numbers occurred very fast compared with the untreated rats (Figure 1). Calculating the maximum generation time (disregarding transfer and the intestinal bacterial dilution rate) for the first day where transconjugants are detected, it would be $\sim$60 and 300 min for the erythromycin-treated and untreated rats, respectively. If a selective growth advantage was the only factor responsible for the increase in transconjugant numbers, the generation time in the erythromycin-treated rats should be well below 1 h when the bacterial dilution rate is taken into account. Therefore, it is speculated that an increased plasmid transfer rate has contributed to the fast increase in transconjugant numbers in the erythromycin-treated rats.

There are only few reports on the effect of erythromycin on transfer of erythromycin resistance determinants. Nevertheless, the presented results are in good agreement with those of Igimi *et al.*,[22] who examined the transfer of pAMβ1 from *L. lactis* to bacteria belonging to the 'Schaedler flora' in previously germ-free

mice. When treatment with erythromycin was initiated, an increase in *E. faecalis* harbouring pAMβ1 from below detection limit to $\sim 10^7$ cfu/g faeces within 1 day was observed.[22] Likewise, Morelli *et al.*[23] observed an improved plasmid dissemination efficiency from 10^{-7} to 10^{-4} transconjugants/donor after initiation of erythromycin treatment in gnotobiotic mice colonized with pAMβ1-bearing *Lactobacillus reuteri* donor strain and *E. faecalis* recipient. However, to our knowledge, there are no reports describing selection-induced transfer by erythromycin, as is known for conjugative transposons carrying *tet*(M).[24–26]

Also *in vitro*, an effect of low concentration of erythromycin was detected, which was abolished at high concentration. Addition of 0.5 mg/L erythromycin to the *in vitro* mating plates resulted in a factor of 30 increase in the ratio of transconjugants to recipients compared with the control, whereas 5 mg/L erythromycin in the mating plates resulted in a ratio similar to the control (Table 2). The selection for transconjugants by low concentrations of erythromycin in the mating medium could again be due to two things: (i) increased transfer frequency and/or (ii) a growth advantage of transconjugants. We believe that inhibition of the recipient at higher erythromycin concentrations restricts transfer, as reported previously,[27,28] and thus counteracts the selective effects of erythromycin on transconjugants exerted at lower concentrations. In contrast to the results described here, Morelli *et al.*[23] did not see any effect of adding 1 mg/L erythromycin to the mating plates in the transfer of pAMβ1 from *L. reuteri* to *E. faecalis* JH2SS. These diverging results could possibly be explained by (i) the donor/plasmid examined by Morelli *et al.* did not respond to erythromycin by induction of transfer or (ii) the selection for transconjugants over recipients depends on their respective growth rates, which are affected by the specific R-plasmid and the erythromycin concentration. The latter has, for instance, been shown for different R-plasmids, where the equilibrium between susceptible and resistant populations in the gut was changed with different concentrations of the antibiotic.[29]

In the gnotobiotic gut, the transconjugants persisted in stable numbers for at least 12 days after the end of erythromycin treatment. This indicates that either the resistance plasmid did not represent a noteworthy competitive disadvantage in the absence of antibiotic selection and/or the plasmid had a high rate of transfer. In other *in vivo* studies looking at transconjugant persistence without selective pressure, both relatively stable[30–33] and rapidly decreasing,[34,35] transconjugant populations have been reported. The fact that the recipient population was well established in the intestine prior to donor introduction could be an important competitive parameter for the newly developed transconjugant population. Thus, the recipients who acquire the plasmid do not need to compete for available niches since they are already attached to the intestinal mucosa.

The ability to conjugate is another parameter shown to be important for the maintenance of a plasmid-carrying population in the gut.[36] Whether secondary transfer of the *erm*(B) plasmid from transconjugants to recipients contributed to the stability of the plasmid in the gnotobiotic model is not known. It was tried to elucidate the potential of the transconjugants to act as new donors by *in vitro* mating to an isogenic recipient. Although no transfer was observed by this method, it cannot be excluded that the transfer can take place in the gastrointestinal tract. Nevertheless, it is believed that the *L. plantarum* donor strain harbours genes for conjugation of the small mobilizable *erm*(B) plasmid either on the chromosome or on a larger plasmid, which either are not co-transferred or are co-transferred but not maintained in the *E. faecalis* recipient.

In the gnotobiotic rats, a frequency of up to 1 transconjugant/recipient was observed, demonstrating that the *L. plantarum* strain maintains an active transfer apparatus after passage of the stomach and upper intestine. In contrast, no transconjugants were detected in the more complex model using streptomycin-treated mice, where transfer was assessed in the presence of a surrounding microbiota. This indicates that the presence of the indigenous intestinal bacteria efficiently reduces the formation of effective mating pairs. This finding is in agreement with previous investigations that have found a lower transfer rate in animals with a colonization barrier than in a gnotobiotic intestinal environment.[37] However, lacking detection of transfer in the streptomycin-treated mice does not exclude that transfer took place, but suggests that it was a rare event.

Despite the fact that a relatively low transfer frequency between *L. plantarum* and *E. faecalis* was observed *in vitro*, this study shows that a surprisingly high concentration of transconjugants can be obtained in the gastrointestinal tract of gnotobiotic rats. This concentration can be considerably further raised during treatment with erythromycin.

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Transparency declarations

None to declare.

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Paper III

Louise Feld, Karin Hammer and Andrea Wilcks. Characterization of a small erythromycin resistance plasmid pLFE1 from the food-isolate *Lactobacillus plantarum* M345.
In preparation.

Title: Characterization of a small erythromycin resistance plasmid pLFE1 from the food-isolate *Lactobacillus plantarum* M345

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Abstract

This paper reports the complete 4031 bp nucleotide sequence of the small erythromycin resistance plasmid pLFE1 derived from the raw-milk cheese isolate *Lactobacillus plantarum* M345. Analysis of the sequence revealed the coding regions for the erythromycin resistance determinant *erm*(B) and a small erythromycin leader peptide suggested to be involved in regulation of *erm*(B). A replication initiation protein *repB* was identified belonging to the RepB proteins of the pMV158 family of rolling-circle replicating (RCR) plasmids. Two elements, the transcriptional repressor protein *copG* and a small countertranscribed RNA (ctRNA), typically involved in replication control within this family were also found. A putative replication initiation site including a single-strand origin (*sso*) - like region succeeded by a characteristic pMV158 family double-strand origin (*dso*) was located upstream of the replication region. Moreover, we suggest the presence of a secondary replication initiation site including an *sso* and a pC194-type *dso.* An open reading frame (ORF) preceded by a typical origin of transfer (*oriT)* site and coding for a putative truncated mobilization (Mob) protein with a size of 83 aa was detected. The putative *mob* gene showed large similarity to the N-terminal region of the pMV158 family of Pre/Mob proteins, but was much smaller than other proteins of this family. We therefore suggest that the Mob function in pLFE1 is supplied in trans from another plasmid present in *L. plantarum* M345.

Filtermating experiments were performed in order to determine the ability of pLFE1 to be transferred to and replicate in different species of Gram-positive bacteria. pLFE1 was shown to have a broad host-range with transconjugants obtained from *Lactobacillus rhamnosus, Lactococcus lactis, Listeria innocua*, the opportunistic pathogen *Enterococcus faecalis* and the pathogen *Listeria monocytogenes*.

Introduction

Lactobacillus plantarum is a species of substantial industrial importance and has been used for centuries as starter culture in the manufacture of a broad selection of food products both for human and animal consumption. In addition, many strains of *L. plantarum* are recognized to have a general beneficial health effect, and are thus used as probiotics. *L. plantarum* is often well adapted to survive passage through the upper gastrointestinal tract and has frequently been shown to be one of the dominating *Lactobacillus* species in the human small intestine (Ahrne et al. 1998;Johansson et al. 1993).

Natural resident plasmids are common in *L. plantarum* and several papers have reported the nucleotide sequence of these: pWCFS101, pWCFS102, pWCFS103 (van Kranenburg et al. 2005), p256 (Sorvig et al. 2005), pPB1 (las Rivas et al. 2004), pPLA4 (van Reenen et al. 1998), pA1 (Vujcic and Topisirovic 1993), pLB4 (Bates and Gilbert 1989) and pC30il (Skaugen 1989). However, most plasmids in lactobacilli are small rolling-circle replicating (RCR) cryptic plasmids without any assigned function besides their own replication apparatus and occasionally mobilization genes (Pouwels and Leer 1993). Some documents exist on the occurrence of antibiotic resistance genes harboured on *Lactobacillus* plasmids, however, the number is relatively limited (Axelsson et al. 1988;Fons et al. 1997;Gevers et al. 2003a;Lin et al. 1996;Tannock et al. 1994;Vescovo et al. 1982). Due to the wide environmental distribution of *L. plantarum*, the presence of antibiotic resistance plasmids in this species can be a potential health hazard. If mobile, the plasmids may be transferred to other bacteria within the food chain and in worst-case reach potentially pathogenic bacteria invading the consumer. Transfer of different *L. plantarum* plasmids harbouring resistance genes towards erythromycin and tetracycline has been documented *in vitro* to *Enterococcus faecalis* and *Lactococcus lactis* (Gevers et al. 2003b) and *in vivo* to *E. faecalis* using gnotobiotic rats as a model of the human intestine (Jacobsen et al. 2007).

The present paper reports on the small *L. plantarum* erythromycin resistance plasmid pLFE1, previously shown to transfer at high frequency to *E. faecalis* in the gnotobiotic rat model but not in the more complex streptomycin-treated mice model (Feld et al. 2008). The whole nucleotide sequence of pLFE1 was determined and analysis was carried out in order to learn more about the mechanistic basis for mobilization, potential host-range, incompatibility grouping and expression of antibiotic resistance.

Materials and methods

Bacterial strains, plasmids and culture conditions

The raw-milk cheese isolate *Lactobacillus plantarum* M345 (Feld et al. 2008) previously shown to harbour the erythromycin resistance plasmid pLFE1 was used for plasmid preparation of pLFE1 and as donor in *in vitro* conjugation assays. Strain M345 was grown anaerobic at 37 °C for 24-48 h in de Man, Rogosa and Sharpe (MRS) medium (Oxoid, Hampshire, UK) supplemented with erythromycin when appropriate (for concentrations, see below).

Bacterial strains used as recipients in the conjugation study are listed in Table 1. When appropriate, antibiotics were added to the media at the following final concentrations: 100 μg ml^{-1} or 500 μg ml^{-1} streptomycin (str) for selection of *Bacillis* sp. and *Enterococcus faecalis*, respectively, 500 μg ml^{-1} spectinomycin (spec), 25 μg ml^{-1} tetracycline (tet), 100 μg ml^{-1} rifampicin (rif) and 100 μg ml^{-1} nalidixic acid (nal). Erythromycin was applied at 16 or 50 μg ml^{-1} for selection of transconjugants and donors, respectively.

Sequencing of pLFE1 plasmid

The *erm*(B) gene harboured on plasmid pLFE1 from *L. plantarum* M345 was analysed by sequencing standard PCR products. Plasmids were extracted from the strain as earlier described (Jacobsen et al. 2007) and subsequently used as template in PCR amplification of the *erm*(B) gene, as previously described (Jacobsen et al. 2007). Eight PCR reactions were set up, and the eight products pooled and purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany). The size of the PCR product (424 bp) was confirmed by gel electrophoresis and the sample subsequently sequenced using the ermB-F and ermB-R primers (Jensen et al. 1999). The remaining part of the *L. plantarum* M345 *erm*(B) plasmid was sequenced by primer walking using the plasmid preparation of strain M345 as template. Initially, the primers Lor1 (5'-AGA TGA CTG TCT AAT TCA ATA G-3') and Lor3 (5'-TGC ACA CTC AAG TCT CGA-3') targeting the *erm*(B) gene and reading outwards, were applied. All sequencing reactions were performed at GATC Biotech (Konstanz, Germany).

Verification of pLFE1 structure

A restriction map of pLFE1 was made by digestion with the restriction enzymes *Xcm*I, *Xmn*I, *Ase*I, *Hinc*II, *Bss*SI and *Nde*I. As template, plasmid extraction from a pLFE1-carrying transconjugant isolate previously obtained by conjugation between *L. plantarum* M345 and *E. faecalis* JH2SS was

used (Feld et al. 2008). Analysis of the plasmid profile showed that this isolate contained the pLFE1 plasmid solely. Restriction analysis was performed using restriction enzymes from New England Biolab (Herts, UK) according to the description of the manufacturer. Digested DNA was run by gel electrophoresis on a 1% agarose gel and subsequently stained with ethidium bromide.

The structure of pLFE1 was further confirmed by PCR using primers mob_1F (5'- TGG GTC AAT CGA GAA TAT C-3') binding at position 1213-1231, mob_2F (5'-GAT TTG GTC AAT CGG ACA G-3') binding at position 3444-3462 and mobR (5'-GAA CGC AAA TAT GAG CTT C-3') binding at position 3729-3711 and 1623-1605. As template, plasmid preparations of *L. plantarum* M345 or transconjugants previously obtained from mating between *L. plantarum* M345 and *E. faecalis* JH2SS were used (Feld et al. 2008). PCR mixtures were prepared using 5 µl DNA template, 1 PuReTaq Ready-To-Go PCR bead (Amersham Biosciences, Buckinghamshire, UK) and 20 pmol of each primer in a total volume of 25 µl. Amplification using the primers mob_1F + mobR or mob_2F + mobR should result in a 286 bp and a 411 bp PCR product, respectively. PCR was carried out by initial denaturation at 95 ºC for 10 min followed by 30 cycles of 95 ºC for 45 s, 55 ºC for 45 s and 72 ºC for 2 min. A final extension step at 72 ºC for 10 min ended the programme.

In vitro mating experiment

The ability of plasmid pLFE1 to be transferred to and replicate in different Gram-positive species was assessed using a filter-mating assay. The donor and recipient strains were grown in broth supplemented with the respective antibiotics to an OD_{600} = 0.7-1.0. The bacteria were mixed in equal volumes and vacuum filtered onto sterile filters (HAWP04700). The filters were placed on agar mating plates and incubated overnight at conditions optimal for the recipients (for media and incubation conditions, see Table 1 – however for mating with *Listeria* sp. and *Bacillus* sp., BHI were used as mating medium instead of selective agars). The bacteria were subsequently washed off the filters and suitable dilutions prepared on plates selective for donor, recipients (Table 1) and transconjugants. Transconjugants were selected at conditions identical to their respective recipients except that erythromycin was added to the plates and the plates were incubated for 36h. Controls of separate donor and recipient plates were also prepared. The experiment was performed in triplicates.

Verification of transconjugants

In order to verify that colonies isolated from selective agar plates were true transconjugants and not mutants of either donor or recipient, specific PCR and fingerprinting assays were performed. The presence of the *erm*(B) gene was verified by PCR using primers specific for *erm*(B) as earlier described (Jacobsen et al. 2007). Donor and recipients were included as positive and negative controls, respectively. Random amplified polymorphic DNA (RAPD) technique was used to show similar fingerprints of recipients and transconjugants and differentiate them from the fingerprint of the donor. Template DNA of donor, recipients and transconjugants was prepared as previously described (Jacobsen et al. 2007). The PCR reaction mixtures contained 5 µl DNA template, 1 PuReTaq Ready-To-Go PCR bead (Amersham Biosciences) and 20 pmol of primer OPA-02 (Operon Biotechnologies, Cologne, Germany) in a total volume of 25 µl. The PCR programme was as follows; initial denaturation 94 ºC for 4 min followed by 40 cycles of 94 ºC for 1 min, 32 ºC for 1 min and 72 ºC for 2 min. The program was ended by extension at 72 ºC for 5 min. Isolates of *Listeria monocytogenes* transconjugants were further tested by PCR using primers IntA_C_up (5'-TAG AAG TAG TGT AAA GAG CTA GAT G-3') and IntA_C_down (5'-ATA TAA AGC TTG CGG CCG CTT CTG CAA AAG CAT CAT CTG GAA AA-3') amplifying a 260 bp sequence of the virulence gene *Int*A coding for Internalin A. PCR reaction mixtures contained 5 µl DNA template, 1 PuReTaq Ready-To-Go PCR bead and 20 pmol of each primer IntA_C_up and IntA_C_down in a total volume of 25 µl. The PCR programme was as follows; initial denaturation at 95 ºC for 3 min followed by 30 cycles of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s and finally extension at 72 ºC for 3 min. The donor and recipient were included as negative and positive controls, respectively.

All PCR amplifications were performed in a PTC-225 Peltier Thermal Cycler (MJ Research, Bio-Rad, Waltham, MA, USA). The PCR products were run on a 1.5% agarose gel for 1h and visualized by ethidium bromide staining.

Nucleotide sequence analysis

Analysis of the complete nucleotide sequence for open reading frames (ORF) was performed using the web-based NCBI ORF Finder programme. Database searches and comparisons of DNA sequences or DNA-derived protein sequences were carried out using BLASTN, BLASTP and BLASTX programmes (Altschul et al. 1990). Multiple sequence alignments were made with the ClustalW2 program (Larkin et al. 2007) and alignments of conserved domains of proteins encoded

on pLFE1 was retrieved from the Conserved Domain Database (CDD) (Marchler-Bauer et al. 2007). Secondary structure and calculation of free energy for hairpin formations was determined using the Mfold programme (Zuker 2003).

Nucleotide sequence accession number

The complete nucleotide sequence of pLFE1 (see appendix) will be deposited into GenBank.

Results and Discussion

General features of pLFE1 sequence

The complete nucleotide sequence of plasmid pLFE1 was determined to be 4031 bp in length. The correctness of the deduced pLFE1 nucleotide sequence was verified with single digests using the restriction enzymes shown in Fig. 1 and gel electrophoresis, which confirmed the predicted nucleotide band sizes. The average G + C content of pLFE1 is 34.4%, which is lower than that of the *L. plantarum* chromosome average of 44.5% (Kleerebezem et al. 2003), but within the range described for other small *L. plantarum* plasmids such as pA1 (34.9%) (Vujcic and Topisirovic 1993), pPB1 (37.4%) (las Rivas et al. 2004), p256 (37%), pWCFS101 (39.5%) and pWCFS102 (34.3%) (van Kranenburg et al. 2005). Analysis of the whole nucleotide sequence revealed 16 putative ORFs of at least 33 aa in length and one ORF smaller than 33 aa (Table 2). In a region of 3-12 nucleotides upstream of each of the ORFs, analysis was carried out for detection of ribosome-binding sites (RBS) using the *L. plantarum* RBS sequence GGAGG as consensus (Pouwels and Leer 1993). However, in many cases either no or only poor potential RBS sites could be detected and translational coupling to other putative proteins did not seem plausible. In addition, several of the ORFs showed either very low or no significant similarity to other proteins deposited in the databanks, and altogether these ORFs were considered artefacts.

Replication initiator (RepB) protein

A putative replication initiator (RepB) protein was identified at position 2421-3059 (212 aa) (Fig 1 + Fig. 2), showing high similarity to the RepB proteins of the *L. plantarum* rolling-circle replicating (RCR) plasmid pLB4 (80% identity over 211 aa) (Bates and Gilbert 1989), RCR plasmid pLF1311 from *Lactobacillus fermentum* (82% identity over 206 aa) (Aleshin et al. 1999) and RCR plasmid pBM02 (71% identity over 205 aa) from *L. lactis* subsp. *cremoris* (Sanchez and Mayo 2003). The putative RepB protein showed a conserved domain architecture corresponding to that of Rep_2 plasmid replication proteins (pfam01719) in the Conserved Domain Database (CDD). The sequence included three motifs (Fig. 2 + Fig. 3) typical for plasmid family pMV158 (Ilyina and Koonin 1992). These motifs include the putative binding site for Mg^{++} (motif II), which is thought to be required for nicking activity and the active tyrosine residue (motif III) involved in the catalytic attack of the plasmid DNA during initiation of replication (del Solar et al. 1998;Dyda and Hickman 2003;Ilyina and Koonin 1992).

Plasmid copy number control

A putative *copG* gene (59aa) previously referred to as *repA* was found at position 1914-1735 bp (Fig. 1 + Fig. 2), showing high similarity to *repA* (70% identity over 51 aa) of pLB4 (Bates and Gilbert 1989), *repA* (70% identity over 51 aa) of pLF1311 (Aleshin et al. 1999), *repA* (68% identity over 51 aa) of the *L. lactis* plasmid pAR141 (Raha et al. 2006) and *repA* (69% identity over 46 aa) of pBM02 (Sanchez and Mayo 2003). The putative CopG protein revealed a conserved sequence structure similar to the RHH_1 ribbon-helix-helix family of CopG repressor proteins (pfam01402 in the Conserved Domain Database) (Fig. 4).

CopG is a transcriptional repressor protein that regulates the plasmid copy number in the pMV158 derivative plasmid pLS1 (del Solar et al. 1995;del Solar and Espinosa 1992). By binding to the single *copG-repB* promoter region, CopG prevents host RNA polymerase binding and thus repress the synthesis of the replication initiation protein RepB as well as itself (del Solar et al. 1990). A structure of the replication region comparable to pLS1 has also been revealed in other plasmids belonging to the pMV158 family suggesting that they are controlled in a similar fashion (Bates and Gilbert 1989;Cocconcelli et al. 1996;Raha et al. 2006;Vujcic and Topisirovic 1993). Sequence analysis of pLFE1 indicated a homologous architecture with a putative promoter 18 nucleotides upstream of the *copG* start codon with a –35 (TTGTAT) and –10 (TATAAT) sequence in a distance of 17 base pairs and typical RBS sites located upstream each gene, *copG* and *repB* (Fig. 2). However, no sequence matching the criteria of a 13-bp pseudosymmetric element overlapping the -35 *copG-repB* promoter box could be found. This element was demonstrated to be the target binding site of CopG in pLS1 (del Solar et al. 1990) and homologous sequences have been detected in other pMV158 family plasmids, although their function have not been elucidated (del Solar et al. 2002).

The active DNA binding unit of CopG from plasmid pMV158 has been shown to display a characteristic ribbon-helix-helix (RHH) motif, which is built up around a glycine residue mediating a turn between two α-helices. At conserved positions of the α-helices specific residues are required to maintain the hydrophobic core of the motif (Gomis-Ruth et al. 1998). Within the putative *copG* gene of pLFE1 the presence of a glycine residue and specifically positioned hydrophobic residues were established (Fig. 4).

Apart from CopG, a small countertranscribed RNA (ctRNA) of approximately 50 nucleotides in size has been shown to control the plasmid copy number of pLS1 (del Solar et al. 1995;del Solar et al. 1997). The ctRNA is transcribed in the opposite direction of the mRNA encoding the RepB

protein and thus inhibits translation of RepB by binding to a region of the *copG-repB* mRNA, which includes the RBS of *repB* (del Solar et al. 1997). Similar genetic organizations of ctRNA are present in other members of the pMV158 family. Thus, the coordinate action of the two plasmid-encoded elements CopG and ctRNA has therefore been suggested to be a common mode of plasmid copy number regulation in the pMV158 plasmid family (del Solar and Espinosa 1992). Indeed, sequence analysis of pLFE1 revealed a putative ctRNA encoded on the complementary strand in the region between *copG* and *repB* (Fig. 1 + Fig. 2). Within the amino-terminal region of *repB* a putative promoter consisting of less conserved –10 (TTTCAT) and –35 (TAGGCA) boxes in a distance of 18 nucleotides was detected. A possible transcriptional terminator was found overlapping the carboxyl-terminal end of *copG*. This plausible terminator could be a *rho*-independent site, containing an inverted repeat configuring a potential hairpin structure followed by a T-stretch (depicted by an A-stretch at the complementary strand in Fig. 2).

Origin of replication

In RCR plasmids the double-strand origin (*dso*) is the initiation site of leading-strand synthesis. The *dso* contains a *nic* site, which generally is well conserved within each plasmid family and a binding site showing larger sequence variation (Khan 1997). Within pLFE1 a region of 22 bp (5'-atGGGGGcACTACGACaCCCCC-3') showing high similarity (four mismatches) to the pMV158 family *nic* sites (del Solar et al. 1998) was found 162 bp upstream of *copG* at position 1934-1955 bp (Fig. 1 + Fig. 2). This region showed 100% similarity to the *dso* of both *L. fermentum* (Aleshin et al. 1999) and the shuttle vector pLF14 (unpublished, Accession No. X85436.1). The putative *nic* site of pLFE1 was contained within an inverted repeat (IR5 with a calculated free energy dG = -8.4 kcal/mol) and flanked by a direct repeat (DR) (Fig. 2), which is suggested to be the binding site of RepB. In other RCR plasmids, binding and interaction with RepB has been shown to change the secondary structure resulting in creation of a hairpin, which in turn exposes the *nic* site and thus initiates replication (Khan 1997).

A region of 266 nucleotides immediately upstream of the putative *dso* showed a large potential of palindromic structures (dG = -50.6 kcal/mol) (Fig. 1 + Fig. 2). We propose that this region constitutes the single-strand origin (*sso*) of pLFE1 from where lagging-strand synthesis is initiated. *Sso*s are characterised by substantial secondary structure, but the sequence homology may be very low even between plasmids belonging to the same family (Gruss and Ehrlich 1989;Khan 1997). Comparison of the putative *sso* sequence of pLFE1 with four types of *sso*s (*sso*A, *sso*U, *sso*T and

*sso*W) previously described (Kramer et al. 1999) showed no general sequence similarity (data not shown). However, an RS_B (recombination site) or RS_B-like site (5'-TTTTCGTCGGCATAA-3') was recognized at position 1669-1683 (Fig. 2) at the basis of the stem of a putative hairpin structure (dG for IR1 = -33 kcal/mol). This site has apart from being a recombination site been proposed to be involved with host RNA polymerase binding to the *sso.* The RS_B may therefore be critical for the synthesis of a primer RNA that can be used to initiate lagging-strand replication. The RS_B was first recognized in *sso*A origins, but homologues have been detected in the other types of *sso*s as well (Kramer et al. 1999).

A second region of plasmid pLFE1 potentially containing an origin of replication was found in the non-coding area downstream the putative *mob* gene. A sequence of 20 bp (5'-TtcTTCTTATCTTGATAcTA-3'), which showed high homology (three mismatches) with pC194 family *dso*s was found at position 4009-4028 (Fig. 1) (del Solar et al. 1998;Wu et al. 2007). This sequence showed 100% identity with the putative *dso* of the *L. plantarum* plasmids pLP1 (Bouia et al. 1989) and pC30il (Skaugen 1989) and the *L. lactis* plasmid pWC1 (Pillidge et al. 1996).

Upstream of the putative *dso* another potential *sso* region of 240 nucleotides (Fig. 1), which showed high potential of palindromic structures (dG = -63.2 kcal/mol) was detected. This region contained an RS_B-like sequence 100% identical to the one described above, which also was placed at the basis of the stem of a potential hairpin structure (dG = -35.2 kcal/mol). The sequences pursuing these two identical RS_B-like sequences were 75% identical with the similarity discontinuing upstream of the putative *dso*s (data not shown). Thus, despite the lack of overall sequence homology to any previously known *sso*s, we suggest the presence of two *sso*s within pLFE1. The existence of two functionally intact *sso*s simultaneously present on a single plasmid has earlier been reported for pMV158, which has been shown to contain both an *sso*A and *sso*U type (van der Lelie et al. 1989).

Erythromycin resistance

A putative antibiotic resistance gene *erm*(B) conferring resistance to macrolide, lincosamides and streptogramin (MLS) antibiotics were located at position 625-1362. The *erm*(B) gene encodes a protein 100% identical to the erythromycin ribosome methylase Erm(B) of *E. faecium* (De Leener et al. 2005). The group of Erm(B) proteins is relatively well-conserved showing 98-100% similarity at the nucleotide level (Roberts et al. 1999). Erm(B) enzymes confer resistance by adding one or more methyl groups to a specific adenine residue located in the 23S rRNA subunit thereby preventing binding of the antibiotic (Weisblum 1995a).

A small region (27 aa) was identified 124 nucleotides upstream the *erm*(B) start codon, which at its full length showed 100% identity with MLS leader peptides from a range of mobile elements harboured in different bacterial species. The functional role of MLS leader peptides is to regulate expression of the erythromycin methylase by conformational changes of the secondary structure of the mRNA, which affects the neighbouring region upstream of the methylase (Weisblum 1995b). In the absence of erythromycin, translation of the leader peptide is active. Hereby, it assumes a configuration that positions the RBS site and initial codons of the methylase within the stem of a hairpin structure, thus preventing its translation. However, when erythromycin is present, it binds to and suspends ribosomes involved in translation of the leader peptide. This in turn induces a conformational change and thereby exposes the RBS site of the methylase thereby promoting its translation (Weisblum 1995b).

Several isolates have been reported constitutively to produce erythromycin methylase although putative MLS leader peptides were detected (Gfeller et al. 2003;O'connor et al. 2007). However, the putative MLS leader peptide of pLFE1 is identical to leader peptides from strains of *Streptococcus pneumoniae*, which have been reported functional (Okitsu et al. 2005). Compared to the leader peptide in the *erm*(B) gene of Tn*917* (Shaw and Clewell 1985) the peptide in pLFE1 is only 27 aa i.e. 9 aa shorter due to a single mutation resulting in an earlier stop codon. A previous study suggested that a similar mutation caused an increased level of methylase translation, resulting in an apparently constitutive resistant phenotype. The mutation increased the basal methylase activity by approximately 3.8 fold and the induced activity by approximately 4.9 fold (Oh et al. 1998).

Mobilization region

A truncated recombinase/mobilization (*pre/mob*$_1$) gene (Fig. 1) was found at position 1386-1606 (73 aa), which showed 100% nucleotide identity with the carboxyl-terminal end of the *Lactobacillus sakei* plasmid pYS18 Pre/Mob protein (unpublished, Accession No. EU185047) and 94% nucleotide identity with the Pre/Mob protein of *L. lactis* subsp. *lactis* plasmid pK214 (unpublished, Accession No. YP_001429536). However, despite the presence of an ATG start codon, no potential RBS or transcriptional coupling could be detected (Table 2) thus rendering transcription of this protein unlikely.

In a different region of pLFE1 a second truncated *pre/mob*$_2$ gene was found at position 3312-3563 bp (83 aa). A strong RBS site (AGGAG) was located seven nucleotides upstream of the ATG start codon and a possible promoter was found with a -35 box (TTACGA) and a -10 box (TATACT) in a

distance of 17 nucleotides (Fig. 5). The *pre/mob*$_2$ gene showed 94% nucleotide identity with the amino-terminal end of the Pre/Mob protein of pK214 and 81% nucleotide identity with the Pre/Mob protein of pYS18. Sixtyone nucleotides downstream of the *pre/mob*$_2$ stop codon a fragment showing 93% nucleotide identity with the carboxyl-terminal end of the pK214 Pre/Mob protein and 96% nucleotide identity with the Pre/Mob protein of pYS18 was found (Fig. 5). However, no start codon or putative RBS could be identified for this small fragment.

The odd sequence structure of pLFE1 with several truncated *pre/mob* genes or gene fragments was verified by PCR using primers mob$_1$F, mob$_2$F and mobR (data not shown). The mob$_2$F + mobR primers were specifically designed to confirm the short distance of 61 nucleotides and thus lack of a middle region of the *pre/mob* gene between the amino-terminal *pre/mob*$_2$ gene and the carboxyl-terminal *pre/mob* fragment. Mob$_1$F + mobR were designed to confirm the spatial separation of *pre/mob*$_1$ and *pre/mob*$_2$ with the former placed downstream of *erm*(B).

The Pre/Mob proteins of pK214 and pYS18 belongs to the pMV158 family of Pre/Mob proteins that functions both in mobilization and recombination (Pre for plasmid recombination enzyme) (Francia et al. 2004). The Pre/Mob family contains within the amino-terminal region three highly conserved motifs considered to form part of the catalytic centre of the relaxase enzyme. In the *pre/mob*$_2$ region of pLFE1 only two of the three motifs could be identified (Fig. 5). Additionally, the size of the putative Pre/mob$_2$ is only 83 aa in contrast to other considerably larger Pre/Mob proteins (approximately 350-500 aa) belonging to this family (Gennaro et al. 1987;Josson et al. 1990;las Rivas et al. 2004;Somkuti and Steinberg 2007).

Mobilization proteins normally demonstrate relaxase activity and are thus essential in preparation of the plasmid for transfer (Francia et al. 2004). In plasmid pMV158 the Pre/Mob protein has been shown to nick supercoiled plasmid DNA at the origin of transfer (*oriT*) (Guzman and Espinosa 1997) and without the presence of an intact Pre/Mob protein conjugal transfer was suspended (Priebe and Lacks 1989;van der Lelie et al. 1990). Since pLFE1 has been shown transferable but does not seem to contain an intact Pre/Mob protein, we suggest that it must be supplied in *trans* in *L. plantarum* M345. This organization has been shown functional in *Bacillus*, where transfer-deficient Mob$^-$ hybrid plasmid constructs of pUB110 and pBC16 could be mobilized by complementation of a Mob protein in *trans* (Selinger et al. 1990).

Apart from relaxase activity, which could be provided in *trans*, the presence of an *oriT* region in *cis* has been shown to be a minimal requirement for mobilization of plasmids such as pUB110 and pBC16 (Selinger et al. 1990). The *oriT* site is very similar among members of the pMV158 family

and is located upstream of the *mob* gene, overlapping its promoter. *OriT* has a conserved hairpin structure with an IR of 7-10 nucleotides forming the stem and a loop of usually 6 nucleotides presenting the *nic* site (Francia et al. 2004;Guzman and Espinosa 1997). A putative *oriT* site matching these criteria was identified in pLFE1 at position 3257-3276, 34 nucleotides upstream of the *pre/mob*$_2$ start codon. A putative –10 promoter box was embedded within this sequence and an IR consisting of 8 nucleotides interrupted by 6 nucleotides was also recognized (Fig. 5).

Plasmid recombination

Apart from being the origin of transfer, *oriT* also functions as a putative recombination site (RS_A). The plasmid-encoded Pre/Mob protein mediates site-specific recombination at RS_A in *trans* (Gennaro et al. 1987). Analysis of several plasmids has shown a marked sequence divergence at the RS_A site with the one side showing significant homology to one plasmid and the other side showing homology to a second plasmid – thus indicating development by recombination of the plasmids at their respective RS_A sites (Hauschild et al. 2005;van der Lelie et al. 1989). In pLFE1 a similar organization was detected where the sequence including and upstream RS_A exhibits 96% nucleotide identity with a region of the *L. lactis* plasmid pK214, yet the sequence immediately downstream RS_A presents no significant similarity to pK214 but 96% identity with regions of the *L. plantarum* plasmids pPLA4 (van Reenen et al. 1998) and pPB1 (las Rivas et al. 2004) (data not shown). However, pLFE1 has a very unusual sequence structure amongst others with several interrupted *pre/mob* regions suggesting that pLFE is the product of several relatively recent recombination events. The presence of *pre/mob*$_1$ could be the result of recombination with pYS18, since a fragment of 308 nucleotides including *pre/mob*$_1$ shows 100% nucleotide identity with pYS18. The similarity starts with G in the putative ATG start codon and the complete identity continues ten nucleotides downstream of the RS_B recombination site (data not shown).

In vitro mating experiment

A filter-mating assay was used to assess the ability of pLFE1 to be conjugated to and replicate in different Gram-positive bacteria. Potential pLFE1-carrying transconjugants was isolated on selective agar plates from matings with *E. faecalis*, *L. rhamnosus*, *L. lactis*, *L. monocytogenes* and *L. innocua* recipients. These isolates were confirmed to be true transconjugants by PCR using specific primers and by RAPD fingerprinting (data not shown). No transfer was detected to the recipients *B. subtilis* and *B. thuringiensis* under the experimental conditions applied (data not

shown). However, transfer and maintenance of pLFE1 in the *Bacillus* species cannot be excluded from the present results, but may be possible under different conditions.

Host-range of pLFE1

The *in vitro* mating experiment included in this study established the ability of a broad range of Gram-positive species to function as hosts of pLFE1. An important factor for the stable maintenance of RCR plasmids in a host is the presence of a compatible *sso*, since lack of this will result in accumulation of single-stranded plasmid DNA (Leer et al. 1992;van der Lelie et al. 1989) and subsequently segregation (Gruss and Ehrlich 1989). The efficiency of the host RNA polymerase to bind to the plasmid *sso* promoter sequence has earlier been suggested as a key factor deciding the compatibility between the host and the plasmid (Kramer et al. 1998;Kramer et al. 1999). In pLFE1, the observed broad host-range could imply the presence of an *sso*U type of *sso*, since the other types of *sso*s seem to function optimally only in their natural hosts, possibly due to a more specific binding site. In contrast, *sso*U has been isolated from a broad range of Gram-positive bacteria such as *B. subtilis*, *Staphylococcus aureus*, *S. pneumoniae* and *L. lactis* (Kramer et al. 1999).

Analysis of the pLFE1 nucleotide sequence failed to detect the presence of a typical *mob* gene, but rather revealed a number of truncated genes or fragments with similarity to the pMV158 family of recombination and mobilization proteins. Members of this family have been shown to replicate both in Gram-positive and Gram-negative hosts, and mobilization of these can be facilitated by a wide range of helper plasmids such as the Gram-positive conjugative plasmids pIP501, pAMβ1, pLS20, pXO11, pXO12 and pAD1 as well as Gram-negative broad host-range plasmids belonging to incompatibility group IncW, IncF and IncP and conjugative transposons like Tn*916*, Tn*925* and Tn*1545* (for a review, see (Francia et al. 2004). However, whether the *mob* genes found in pLFE1 are functional and can provide the relaxase activity needed to initiate plasmid transfer is uncertain and requires further study. However, we suggest that the *mob* function of pLFE1 is supplied in *trans* from another plasmid present in the *L. plantarum* M345 host. If a functional *mob* gene is absent in pLFE1, it significantly reduces the probability of dissemination of pLFE1 from hosts other than *L. plantarum* M345. Hence, in order to sustain transfer, both a *mob* gene compatible with the pMV158 type of *oriT* and a *tra* gene facilitating the mating channel between the new host and recipient will be required in *trans.*

Conclusion

In this paper we have analysed the nucleotide sequence of plasmid pLFE1 and found two regions containing a putative double-strand origin and single-strand origin suggesting that pLFE1 replicates via a rolling-circle mechanism. Furthermore, analysis of the replication region places pLFE1 in the pMV158 family of RCR plasmids. We have shown that PLFE1 has a broad-host range, however the presence of a typical *mob* gene could not be confirmed. Instead, several truncated genes with similarity to the pMV158 family of Pre/Mob proteins were detected. Analysis of the Mob function in pLFE1 requires further study.

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Recipient strains	Resistances	Recipient selection	Source or reference
Enterococcus faecalis OG1SS	Str^r, $spec^r$	BHI + str + spec, 37 C, aerob, 24-48h	(Franke and Clewell 1981)
Lactobacillus rhamnosus VTT E-042730	Tet^r	MRS + tet, 30 C, anaerob, 36h	VTT
Lactococcus lactis BU2-60	Str^r, Rif^r	GM17 + rif + str, 30 C, aerob, 24-48h	(Neve et al. 1984)
Listeria monocytogenes LO28	Nal^r	Palcam + nal, 37 C, aerob, 24-48h	(Vicente et al. 1985)
Listeria innocua DSM 20649	Nal^r	palcam + nal, 37 C, aerob, 24-48h	DSMZ
Bacillus subtilis AW59	Str^r	BSA agar + str, 30 C, aerob, 24-48h	(Wilcks, A., unpublished data)
Bacillus thuringiensis GBJ01	Str^r	BSA agar + str, 30 C, aerob, 24-48h	(Jensen et al. 1995)

Table 1: BHI - Brain Heart Infusion Medium; GM17 - M17 Medium supplemented with 0.5% glucose; Palcam Agar Base supplemented with Palcam Selective Supplement; BSA - *Bacillus cereus* Selective Agar Base supplemented with *Bacillus cereus* Selective Supplement and Egg Yolk Emulsion. All media were purchased from Oxoid and prepared according to manufacturers instructions. DSMZ: German Collection of Microorganisms and Cell Cultures. VTT Culture Collection, Finland.

Name	strand	Size (aa)	Position (bp)	Mol% G + C	RBS	% aa identity	Best BLAST match	Accession no.
***Erm*(B) leader peptide**	**+**	**27**	**417-500**	**29.8**	**GGAGG**	**100 (27 aa)**	**MLS leader peptide, *Streptococcus pneumoniae*, transposon Tn6003**	**AM410044**
***Erm*(B)**	**+**	**245**	**625-1362**	**33.1**	**GGAGA**	**100 (245 aa)**	**ErmB, Erythromycin ribosome methylase of *E. faecium* clone 1**	**AAX12187**
ORF 3	+	34	1166-1270		–		no significant similarity	
ORF 4	+	64	1307-1501	31.8	GTACC	73 (34 aa)	hypothetical protein, *Streptococcus cristatus*	AAY63933
pre/mob$_1$	+	73	1384-1605	33.8	_	100 (72 aa)	Mob, mobilization protein of *Lactobacillus sakei* plasmid pYS18	ABW71679
CopG	**+**	**59**	**2118-2297**	**29.4**	**AGAGA**	**70 (51 aa)**	**RepA, plasmid copy number control protein of *L. plantarum* RCR plasmid pLB4**	**P20044**
ORF 7	+	42	2272-2400		CAAGC		no significant similarity	
RepB	**+**	**212**	**2421-3059**	**33.8**	**GGAAG**	**80 (211 aa)**	**RepB, replication initiation protein of *L. plantarum* RCR plasmid pLB4**	**P20045**
ORF 9	+	33	3077-3178		–		no significant similarity	
***pre/mob*$_2$**	**+**	**83**	**3312-3563**	**32.5**	**AGGAG**	**95 (83 aa)**	**Mob, mobilization protein of *L. lactis* subsp. *lactis* plasmid pK214**	**YP_001429536**
ORF 11	+	33	3442-3543		–		no significant similarity	
ORF 12	+	35	3800-3907		AGCGT		no significant similarity	
ORF 13	–	59	338-159		TAAGA	31 (61 aa)	C-terminal domain containing protein, *Tetrahymena termophila*	XP_001013113
ORF 14	–	72	1560-1342		_	33 (56 aa)	hypothetical protein, *Paramecium tetraurelia*	XP_001449055
ORF 15	–	41	1735-1610		GCAAG		no significant similarity	
ORF 16	–	36	1763-1653		GCAAA		no significant similarity	
ORF 17	–	65	3176-2979		AGCGC	34 (58 aa)	rCG54873, isoform CRA_a, *Rattus norvegicus*	EDL98520

Table 2: Description of putative ORFs in pLFE1. ORFs marked in bold have a high probability of translation as predicted from RBS sites and potential translational coupling.

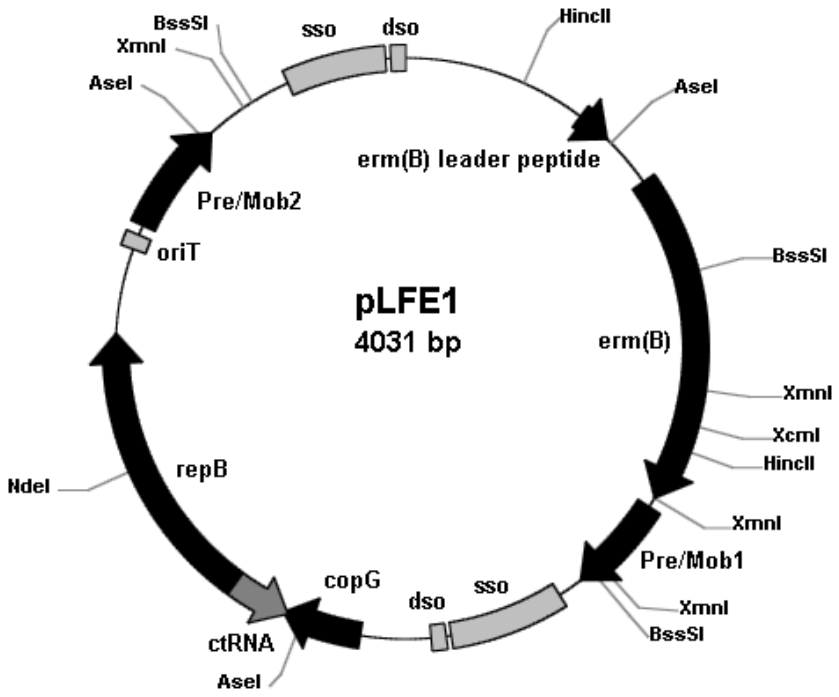
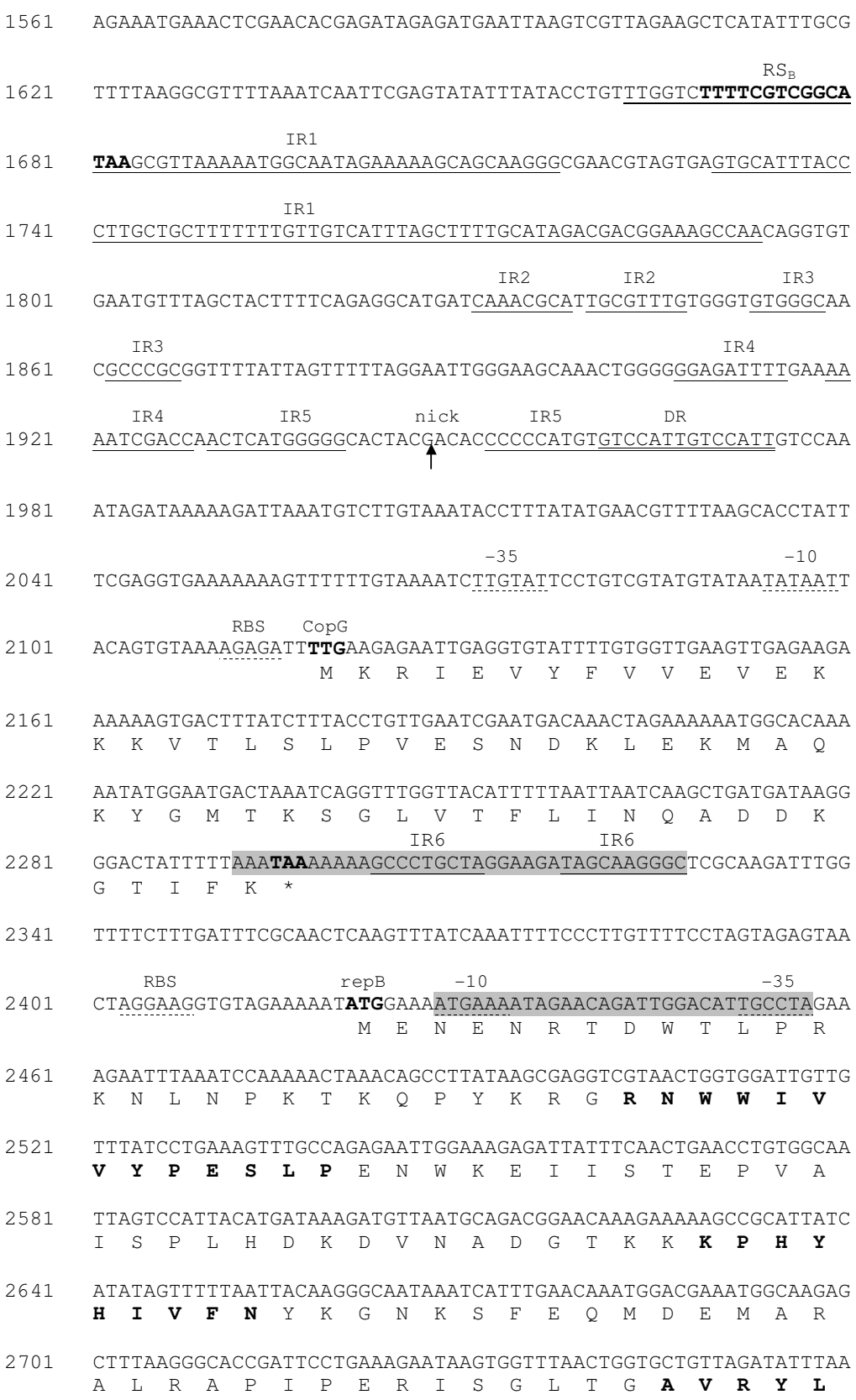


Fig. 1: Map of plasmid pLFE1 showing elements described in the text and restriction sites for enzymes used for nucleotide sequence verification.


1561 AGAAATGAAACTCGAACACGAGATAGAGATGAATTAAGTCGTTAGAAGCTCATATTTGCG
RS$_B$
1621 TTTTAAGGCGTTTTAAATCAATTCGAGTATATTTATACCTGTTTGGTCTTTTCGTCGGCA
IR1
1681 TAAGCGTTAAAAATGGCAATAGAAAAAGCAGCAAGGGCGAACGTAGTGAGTGCATTTACC
IR1
1741 CTTGCTGCTTTTTTTGTTGTCATTTAGCTTTTGCATAGACGACGGAAAGCCAACAGGTGT
IR2 IR2 IR3
1801 GAATGTTTAGCTACTTTTCAGAGGCATGATCAAACGCATTGCGTTTGTGGGTGTGGGCAA
IR3 IR4
1861 CGCCCGCGGTTTTATTAGTTTTTAGGAATTGGGAAGCAAACTGGGGGGGAGATTTTGAAAA
IR4 IR5 nick IR5 DR
1921 AATCGACCAACTCATGGGGGCACTACGACACCCCCCATGTGTCCATTGTCCATTGTCCAA
1981 ATAGATAAAAAGATTAAATGTCTTGTAAATACCTTTATATGAACGTTTTAAGCACCTATT
-35 -10
2041 TCGAGGTGAAAAAAAGTTTTTTGTAAAATCTTGTATTCCTGTCGTATGTATAATATAATT
RBS CopG
2101 ACAGTGTAAAAGAGATTTTGAAGAGAATTGAGGTGTATTTTGTGGTTGAAGTTGAGAAGA
M K R I E V Y F V V E V E K
2161 AAAAAGTGACTTTATCTTTACCTGTTGAATCGAATGACAAACTAGAAAAAATGGCACAAA
K K V T L S L P V E S N D K L E K M A Q
2221 AATATGGAATGACTAAATCAGGTTTGGTTACATTTTTAATTAATCAAGCTGATGATAAGG
K Y G M T K S G L V T F L I N Q A D D K
IR6 IR6
2281 GGACTATTTTTAAATAAAAAAGCCCTGCTAGGAAGATAGCAAGGGCTCGCAAGATTTGG
G T I F K *
2341 TTTTCTTTGATTTCGCAACTCAAGTTTATCAAATTTTCCCTTGTTTTCCTAGTAGAGTAA
RBS repB -10 -35
2401 CTAGGAAGGTGTAGAAAAATATGGAAAATGAAAATAGAACAGATTGGACATTGCCTAGAA
M E N E N R T D W T L P R
2461 AGAATTTAAATCCAAAAACTAAACAGCCTTATAAGCGAGGTCGTAACTGGTGGATTGTTG
K N L N P K T K Q P Y K R G R N W W I V
2521 TTTATCCTGAAAGTTTGCCAGAGAATTGGAAAGAGATTATTTCAACTGAACCTGTGGCAA
V Y P E S L P E N W K E I I S T E P V A
2581 TTAGTCCATTACATGATAAAGATGTTAATGCAGACGGAACAAAGAAAAAGCCGCATTATC
I S P L H D K D V N A D G T K K K P H Y
2641 ATATAGTTTTTAATTACAAGGGCAATAAATCATTTGAACAAATGGACGAAATGGCAAGAG
H I V F N Y K G N K S F E Q M D E M A R
2701 CTTTAAGGGCACCGATTCCTGAAAGAATAAGTGGTTTAACTGGTGCTGTTAGATATTTAA
A L R A P I P E R I S G L T G A V R Y L

```
2761   CGCATATGGATAATCCTGAAAAGTATCAATACGATAATACAGAAATACAAGTGTTTGGTG
       T  H  M  D  N  P  E  K  Y  Q  Y  D  N  T  E  I  Q  V  F  G

2821   GATTTGACCTTGAAAGTTGTTTAGCGTTATCTACTGGTGATAAAAGACAAGCGTTAAAAG
       G  F  D  L  E  S  C  L  A  L  S  T  G  D  K  R  Q  A  L  K

2881   AAATGCTTGGTTTTATTTCGGATAACAATATTATGCATTTAAAAGATTTTGCTGATTATT
       E  M  L  G  F  I  S  D  N  N  I  M  H  L  K  D  F  A  D  Y

2941   GTATGTCTGACCGAGCTCCTGCTGGTTGGTTCGAATTGCTAACAGAGAGGAATACTCTTT
       C  M  S  D  R  A  P  A  G  W  F  E  L  L  T  E  R  N  T  L

3001   TTATAAAAGAGTACATAAAATCGAATTGGCAAAAAGAAAACCAAGTTTATAAAGAGTGAG
       F  I  K  E  Y  I  K  S  N  W  Q  K  E  N  Q  V  Y  K  E  *
```

Fig. 2: Detailed DNA sequence of pLFE1 replication region. Inverted repeats (IR) are underlined and direct repeats (DR) are double underlined. The RS_B-region of the putative *sso* is shown in bold and the *dso nic* site is indicated by an arrow. The deduced amino acid sequences of CopG and RepB are shown and putative –35 and –10 promoter boxes and ribosome binding sites (RBS) are underlined with a slashed line. The start and stop codons are marked in bold as are the three conserved motifs of repB. The putative promoter and terminator region of the ctRNA are shaded.

		Motif I		Motif II		Motif III
pLFE1	29	RNWWIVVYPESLP	71	KP-HYHIVFN	109	AVRYLTHM
pWV01	10	RNFGFLLYPDSIP	66	KKPHYHVIYI	109	SYEYLTHE
pCI411	17	RNWSWIVYPESAP	62	KP-HWHIIIS	100	SVQYLWHR
pLA106	13	RNWTFVLYPESAT	57	KP-HWHIAMF	95	MIRYFAHM
pE194	19	RNWTFVLYPESAK	63	KE-HYHILVM	101	LVRYMLHM
pLB4	29	RNWGIVVYPESLP	71	KS-HYHLVLN	109	AVRYLTHM
pHPK255	27	KAFGFIIYPESAT	62	KP-HFHAIIV	114	AYEYFTHS
pHP489	43	RHWTILIYPESVN	89	KP-HHHLLLV	129	FYEYLTHK
pA1	13	RDWTFIVYPESAP	57	KS-HYHILLT	95	FSVVIWHI
pPSC22	10	RNFGFLLYPDSIP	66	KKPHYHVIYI	109	SYEYLTHE

Fig. 3: Multiple sequence alignment of RepB proteins belonging to Rep_2 plasmid replication proteins (pfam01719) in the conserved domain database (CDD). Motifs typical of plasmid family pMV158 are shown according to (Ilyina and Koonin 1992). Numbers to the left indicate the position of the motifs in each plasmid.

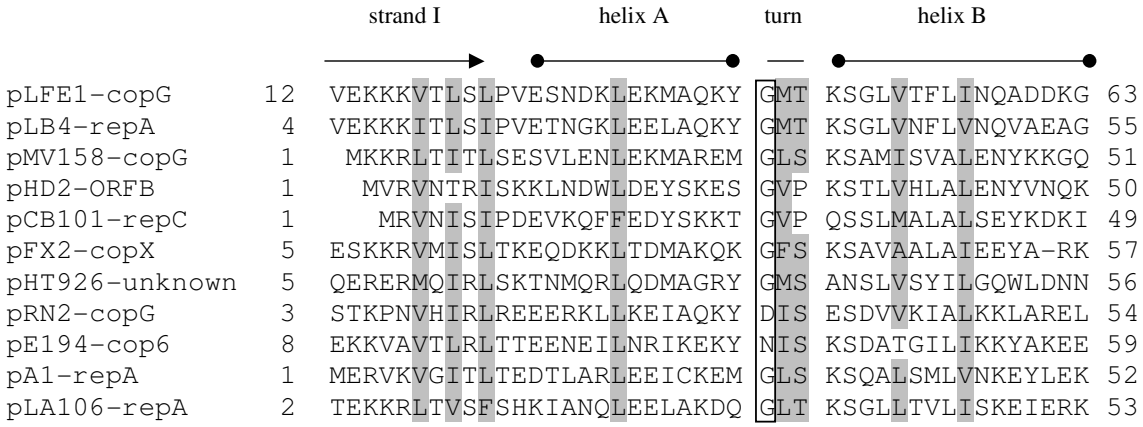


Fig. 4: Multiple sequence alignment of CopG proteins belonging to RHH_1 ribbon-helix-helix CopG repressor proteins (pfam01402) in the conserved domain database (CDD). The glycine residues mediating the turn between the two helices are framed and grey markings are conserved positions with hydrophobic residues (after (Gomis-Ruth et al. 1998). Numbers to the left and right indicate the aligned start and end position of the protein sequences.

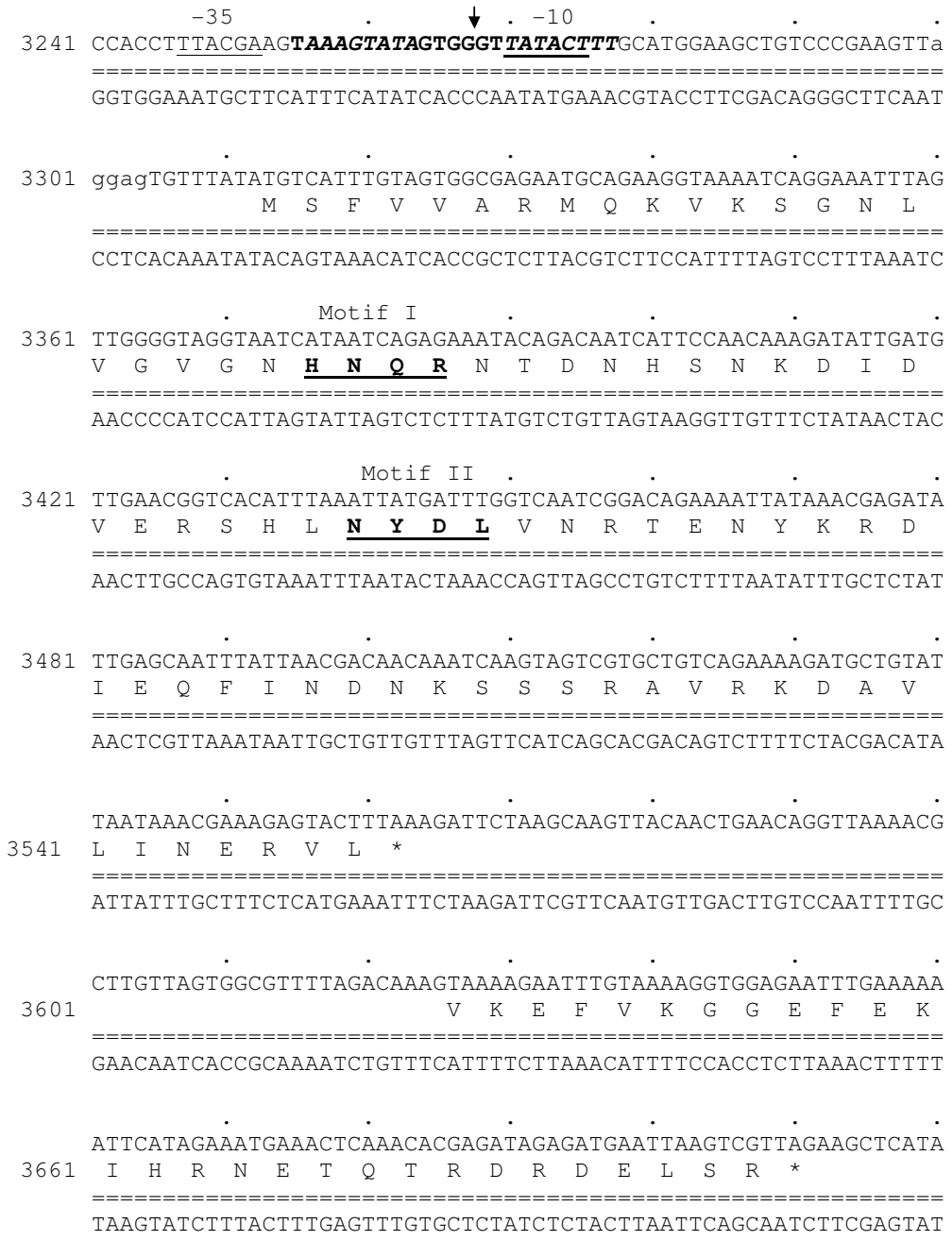


Fig. 5: Sequence analysis of the putative *pre/mob*$_2$ region of pLFE1. The deduced amino acid sequence and the two conserved motifs are indicated as is the amino acid sequence corresponding to the small *pre/mob* carboxyl-terminal fragment. The putative RBS is indicated in small letters and predicted –10 and –35 promoter box sequences are underlined. The nucleotide sequence written in bold is the potential *oriT* region, which includes the IR forming the hairpin structure and the *nic* site, indicated in italic and with an arrow, respectively.

6. Conclusions and discussion

The *Lactobacillus plantarum* strains chosen for testing in this project were naturally occurring wild-type strains recently isolated from Belgian fermented sausages and from a French raw-milk cheese. The strains harboured transferable plasmids conferring resistance to the important human drugs tetracycline and erythromycin. This work includes data documenting transfer of these native plasmids to *Enterococcus faecalis* - an opportunistic pathogen and a common inhabitant of the microbiota in the human gastrointestinal tract. Earlier studies have reported antibiotic resistance gene transfer from *Lactobacillus reuteri* strains to *Enterococcus faecium* in similar environments. However, these have focused on transfer of the broad-host-range conjugative plasmid pAMβ1 originating from *Streptococcus*, which was introduced to the *Lactobacillus* donors (McConnell et al. 1991;Morelli et al. 1988). The present results therefore add important aspects to assessment of antibiotic resistance dissemination from lactobacilli in the food chain, namely wild-type plasmids and naturally occurring bacteria.

Different transfer potentials were observed for the three model donor strains in the GI tract using di-associated rats as models. However, the extent of dissemination *in vivo* did not correlate with transfer frequencies observed in filter-mating experiments. Higher numbers of transconjugants were namely isolated from faecal samples using the *L. plantarum* M345 donor strain with lower *in vitro* transfer frequency compared to *L. plantarum* strain DG507 and DG522 with higher *in vitro* frequencies. These results not surprisingly indicate that *in vitro* mating provides invalid estimates of the transfer potential *in vivo*. This finding may reflect a number of intestinal factors influencing the donor strain as well as the plasmid itself, recipients and transconjugants and thereby the transfer rate in the gastrointestinal environment. Yet, the three donor strains attained different population densities *in vivo*, possibly accounting for some of the observed plasmid dissemination divergences. The background for this could be variable adaptability of the donors to the gastrointestinal environment. Although the three donor strains reached different densities in the GI tract, their numbers however, seemed to remain relatively stable without re-administration. Strain *L. plantarum* M345 decreased approximately one log unit soon after last administration and thereafter stayed constant for 12 days before the experiment was ended. The stability of strain DG507 and DG522 was only observed for three days without donor administration, but lack of administration during this time period did not alter population densities. In all cases, transconjugants also maintained stable population sizes throughout experiments, once their numbers had increased to specific levels.

Treatment with erythromycin at concentrations intended to simulate clinical doses or 1/10 of clinical doses resulted in a practically complete conversion of recipients into transconjugants when using *L. plantarum* M345 as donor in *in vivo* studies. The very fast "turn-over" of the recipient population suggested an increased transfer frequency induced by erythromycin. Hence, spread of plasmid pLFE1 by vertical dissemination (i.e. by selection for the transconjugants and growth at expense of the recipients) was considered to have longer time perspectives than observed for the actual transformation. Filter-mating studies also resulted in higher numbers of transconjugants on media supplemented with medium concentrations of erythromycin compared to negative controls, thus supporting this hypothesis. However, more reliable evidence could be obtained by repetition of the *in vitro* mating study with mating time reduced to a few hours. Thereby the potential bias of selective growth occurring during mating over night could be controlled.
Induction of conjugal transfer by erythromycin is an effect that has not been reported earlier. Such effect considerably enhances the risk associated with plasmid pLFE1. Hence in this situation selective pressure by erythromycin would increase the vertical as well as the horizontal dissemination. Subsequently, more studies are required to confirm or discard this effect and in confirming case elucidate the mechanisms behind.

A relatively high dissemination rate was observed in all experiments conducted on di-associated rats, representing worst-case scenarios for transfer. In contrast, no transfer could be detected to an inoculated *E. faecalis* recipient in a similar experimental set-up using streptomycin-treated mice and *L. plantarum* M345 as donor. Streptomycin-treated mice represent a model with an indigenous microbiota and thus a larger colonization barrier compared to di-associated rats. This situation was reflected by the lower population numbers of inoculated donors and recipients in the streptomycin-treated mice compared to the di-associated rats. Lacking evidence of transfer in the mouse model demonstrates the powerful control exerted by the indigenous microbiota even in a state impaired by streptomycin. However, transfer to the indigenous microbiota was not investigated, and potential recipients adapted to the gastrointestinal environment may have received the plasmid.
The host-range of plasmid pLFE1 was investigated in filter-mating experiments with *L. plantarum* M345 as donor strain. A relatively broad selection of bacterial genera including *E. faecalis*, *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Listeria monocytogenes* and *Listeria innocua* was shown to accept the plasmid. However, transfer of pLFE1 could not be detected by filter-mating from *E. faecalis* transconjugants to isogenic recipients of *E. faecalis.* Secondary transfer of pLFE1

from transconjugants to new recipients cannot be rejected on this basis, since transfer may be feasible either from other transconjugants, under alternative conditions or to other recipients. Yet, analysis of the pLFE1 nucleotide sequence supported the *in vitro* mating results with finding of only a putative truncated and presumably non-functional mobilization protein. Hence, transfer of pLFE1 from other donors than *L. plantarum* M345 may be severely restricted since not only the presence of *tra* genes responsible for mating-pair formation but also a mobilization protein compatible with the pLFE1 origin of transfer is required in *trans*. These functions are apparently present on other mobile elements harboured by strain M345, but a similar constellation may be less likely in other new hosts.

In conclusion, the extent of antibiotic resistance transfer from ingested *Lactobacillus* strains transiently passing the intestine probably will be negligible in most healthy humans. Nevertheless, the risk persists for adaptation of *Lactobacillus*-derived mobile antibiotic resistance determinants into the human gastrointestinal microbiota. The colonization barrier in different human individuals is rather variable and in some individuals, colonization of ingested *Lactobacillus* strains may be allowed. This in turns expands time where interruptions of the microbiota may occur and increases risk of a pathogen receiving the resistance plasmid or conditions selective for the plasmid being imposed. The same scenario can be asserted if an initial transfer event allows integration of the resistance determinant into an existing member of the indigenous microbiota. Once established in a population, the resistance plasmid is often maintained even if it is only as a small sub-population.

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8. Appendix

PLFE1 4031 bp – plasmid isolated from *Lactobacillus plantarum*

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      1        .         .         .         .         .         .
      GGGGTCCCGAGCGCCTACGAGGAATTTGTATCGATAAGAAATAGATTTAAAAATTTCGCT
      ============================================================
      CCCCAGGGCTCGCGGATGCTCCTTAAACATAGCTATTCTTTATCTAAATTTTTAAAGCGA

     61        .         .         .         .         .         .
      GTTATTTTGTACATTTAACTTGACGGTGACATCTCTCTATTGTGAGTTATTAGTGGTACA
      ============================================================
      CAATAAAACATGTAAATTGAACTGCCACTGTAGAGAGATAACACTCAATAATCACCATGT

    121        .         .         .         .         .         .
      GTTTTCAACCGTTTTAATTATAAAAAAGTGGTGCATTTTTAAATTGGCACAAACAGGTAA
      ============================================================
      CAAAAGTTGGCAAAATTAATATTTTTTCACCACGTAAAAATTTAACCGTGTTTGTCCATT

    181        .         .         .         .         .         .
      CGGTTATTGCAGGTGTATTTCTTATCTATGGGTTTAACATGGATTTTATCATTAAAATCA
      ============================================================
      GCCAATAACGTCCACATAAAGAATAGATACCCAAATTGTACCTAAAATAGTAATTTTAGT

    241        .         .         .         .         .         .
      TGAGTATTGTCCGAGAGTGATTGGTCTTGCGTATGGTTAACCCTAAAGTTATGGAAATAA
      ============================================================
      ACTCATAACAGGCTCTCACTAACCAGAACGCATACCAATTGGGATTTCAATACCTTTATT

    301        .         .         .         .         .         .
      GACTTAGAAGCAAACTTAAGAGTGTGTTGATAGTGCATTATCTTAAAATTTTGTATAATA
      ============================================================
      CTGAATCTTCGTTTGAATTCTCACACAACTATCACGTAATAGAATTTTAAAACATATTAT

    361        .         .         .         .         .         .
      GGAATTGAAGTTAAATTAGATGCTAAAAATTTGTAATTAAGAAGGAGGGATTCGTCATGT
      ============================================================
      CCTTAACTTCAATTTAATCTACGATTTTTAAACATTAATTCTTCCTCCCTAAGCAGTACA

    421        .         .         .         .         .         .
      TGGTATTCCAAATGCGTAATGTAGATAAAACATCTACTGTTTTGAAACAGACTAAAAACA
      ============================================================
      ACCATAAGGTTTACGCATTACATCTATTTTGTAGATGACAAAACTTTGTCTGATTTTTGT

    481        .         .         .         .         .         .
      GTGATTACGCAGATAAATAAATACGTTAGATTAATTCCTACCAGTGACTAATCTTATGAC
      ============================================================
      CACTAATGCGTCTATTTATTTATGCAATCTAATTAAGGATGGTCACTGATTAGAATACTG

    541        .         .         .         .         .         .
      TTTTTAAACAGATAACTAAAATTACAAACAAATCGTTTAACTTCTGTATTTGTTTATAGA
      ============================================================
      AAAAATTTGTCTATTGATTTTAATGTTTGTTTAGCAAATTGAAGACATAAACAAATATCT
```

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601        .         .         .         .         .         .
  TGTAATCACTTCAGGAGAGATTACATGAACAAAAATATAAAATATTCTCAAAACTTTTTA
  ============================================================
  ACATTAGTGAAGTCCTCTCTAATGTACTTGTTTTTATATTTTATAAGAGTTTTGAAAAAT

661        .         .         .         .         .         .
  ACGAGTGAAAAAGTACTCAACCAAATAATAAAACAATTGAATTTAAAAGAAACCGATACC
  ============================================================
  TGCTCACTTTTTCATGAGTTGGTTTATTATTTTGTTAACTTAAATTTTCTTTGGCTATGG

721        .         .         .         .         .         .
  GTTTACGAAATTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAA
  ============================================================
  CAAATGCTTTAACCTTGTCCATTTCCCGTAAATTGCTGCTTTGACCGATTTTATTCATTT

781        .         .         .         .         .         .
  CAGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAA
  ============================================================
  GTCCATTGCAGATAACTTAATCTGTCAGTAGATAAGTTGAATAGCAGTCTTTTTAATTTT

841        .         .         .         .         .         .
  CTGAATACTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAA
  ============================================================
  GACTTATGAGCACAGTGAAATTAAGTGGTTCTATAAGATGTCAAAGTTAAGGGATTGTTT

901        .         .         .         .         .         .
  CAGAGGTATAAAATTGTTGGGAATATTCCTTACCATTTAAGCACACAAATTATTAAAAAA
  ============================================================
  GTCTCCATATTTTAACAACCCTTATAAGGAATGGTAAATTCGTGTGTTTAATAATTTTTT

961        .         .         .         .         .         .
  GTGGTTTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAAG
  ============================================================
  CACCAAAAACTTTCGGTACGCAGACTGTAGATAGACTAACAACTTCTTCCTAAGATGTTC

1021       .         .         .         .         .         .
  CGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAG
  ============================================================
  GCATGGAACCTATAAGTGGCTTGTGATCCCAACGAGAACGTGTGAGTTCAGAGCTAAGTC

1081       .         .         .         .         .         .
  CAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTA
  ============================================================
  GTTAACGAATTCGACGGTCGCCTTACGAAAGTAGGATTTGGTTTTCATTTGTCACAGAAT

1141       .         .         .         .         .         .
  ATAAAACTTACCCGCCATACCACAGATGTTCCAGATAAATATTGGAAGCTATATACGTAC
  ============================================================
  TATTTTGAATGGGCGGTATGGTGTCTACAAGGTCTATTTATAACCTTCGATATATGCATG

1201       .         .         .         .         .         .
  TTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTGTTTACTAAAAATCAGTTTCAT
  ============================================================
  AAACAAAGTTTTACCCAGTTAGCTCTTATAGCAGTTGACAAATGATTTTTAGTCAAAGTA

1261       .         .         .         .         .         .
  CAAGCAATGAAACACGCCAAAGTAAACAATTTAAGTACCGTTACTTATGAGCAAGTATTG
  ============================================================
  GTTCGTTACTTTGTGCGGTTTCATTTGTTAAATTCATGGCAATGAATACTCGTTCATAAC
```

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1321        .         .         .         .         .         .
   TCTATTTTTAATAGTTATCTATTATTTAACGGGAGGAAATAATTCTATGAGTCGCTTTTG
   ============================================================
   AGATAAAAATTATCAATAGATAATAAATTGCCCTCCTTTATTAAGATACTCAGCGAAAAC

1381        .         .         .         .         .         .
   TAAATGTTGGAGAAAGAGAATAAATCGCTTAAAAACGAAATTAAGGGCATTTACAGAGGT
   ============================================================
   ATTTACAACCTCTTTCTCTTATTTAGCGAATTTTTGCTTTAATTCCCGTAAATGTCTCCA

1441        .         .         .         .         .         .
   TTTAATCAGTTTATGGAAAATACCCTTGGTGCAACTGTTGGACAAGCTAAAAAGCTCATG
   ============================================================
   AAATTAGTCAAATACCTTTTATGGGAACCACGTTGACAACCTGTTCGATTTTTCGAGTAC

1501        .         .         .         .         .         .
   AATAACCTTGTGAGCGAAATTAAAGAGTTTGTAAAAGGTGGAGAGTTTGAAAAAATTCAT
   ============================================================
   TTATTGGAACACTCGCTTTAATTTCTCAAACATTTTCCACCTCTCAAACTTTTTTAAGTA

1561        .         .         .         .         .         .
   AGAAATGAAACTCGAACACGAGATAGAGATGAATTAAGTCGTTAGAAGCTCATATTTGCG
   ============================================================
   TCTTTACTTTGAGCTTGTGCTCTATCTCTACTTAATTCAGCAATCTTCGAGTATAAACGC

1621        .         .         .         .         .         .
   TTTTAAGGCGTTTTAAATCAATTCGAGTATATTTATACCTGTTTGGTCTTTTCGTCGGCA
   ============================================================
   AAAATTCCGCAAAATTTAGTTAAGCTCATATAAATATGGACAAACCAGAAAAGCAGCCGT

1681        .         .         .         .         .         .
   TAAGCGTTAAAAATGGCAATAGAAAAAGCAGCAAGGGCGAACGTAGTGAGTGCATTTACC
   ============================================================
   ATTCGCAATTTTTACCGTTATCTTTTTCGTCGTTCCCGCTTGCATCACTCACGTAAATGG

1741        .         .         .         .         .         .
   CTTGCTGCTTTTTTTGTTGTCATTTAGCTTTTGCATAGACGACGGAAAGCCAACAGGTGT
   ============================================================
   GAACGACGAAAAAAACAACAGTAAATCGAAAACGTATCTGCTGCCTTTCGGTTGTCCACA

1801        .         .         .         .         .         .
   GAATGTTTAGCTACTTTTCAGAGGCATGATCAAACGCATTGCGTTTGTGGGTGTGGGCAA
   ============================================================
   CTTACAAATCGATGAAAAGTCTCCGTACTAGTTTGCGTAACGCAAACACCCACACCCGTT

1861        .         .         .         .         .         .
   CGCCCGCGGTTTTATTAGTTTTTAGGAATTGGGAAGCAAACTGGGGGGAGATTTTGAAAA
   ============================================================
   GCGGGCGCCAAAATAATCAAAAATCCTTAACCCTTCGTTTGACCCCCCTCTAAAACTTTT

1921        .         .         .         .         .         .
   AATCGACCAACTCATGGGGGCACTACGACACCCCCCATGTGTCCATTGTCCATTGTCCAA
   ============================================================
   TTAGCTGGTTGAGTACCCCCGTGATGCTGTGGGGGGTACACAGGTAACAGGTAACAGGTT
```

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1981        .         .         .         .         .         .
   ATAGATAAAAAGATTAAATGTCTTGTAAATACCTTTATATGAACGTTTTAAGCACCTATT
   ============================================================
   TATCTATTTTTCTAATTTACAGAACATTTATGGAAATATACTTGCAAAATTCGTGGATAA

2041        .         .         .         .         .         .
   TCGAGGTGAAAAAAAGTTTTTTGTAAAATCTTGTATTCCTGTCGTATGTATAATATAATT
   ============================================================
   AGCTCCACTTTTTTTCAAAAAACATTTTAGAACATAAGGACAGCATACATATTATATTAA

2101        .         .         .         .         .         .
   ACAGTGTAAAAGAGATTTTGAAGAGAATTGAGGTGTATTTTGTGGTTGAAGTTGAGAAGA
   ============================================================
   TGTCACATTTTCTCTAAAACTTCTCTTAACTCCACATAAAACACCAACTTCAACTCTTCT

2161        .         .         .         .         .         .
   AAAAAGTGACTTTATCTTTACCTGTTGAATCGAATGACAAACTAGAAAAAATGGCACAAA
   ============================================================
   TTTTTCACTGAAATAGAAATGGACAACTTAGCTTACTGTTTGATCTTTTTTACCGTGTTT

2221        .         .         .         .         .         .
   AATATGGAATGACTAAATCAGGTTTGGTTACATTTTTAATTAATCAAGCTGATGATAAGG
   ============================================================
   TTATACCTTACTGATTTAGTCCAAACCAATGTAAAAATTAATTAGTTCGACTACTATTCC

2281        .         .         .         .         .         .
   GGACTATTTTTAAATAAAAAAAGCCCTGCTAGGAAGATAGCAAGGGCTCGCAAGATTTGG
   ============================================================
   CCTGATAAAAATTTATTTTTTTCGGGACGATCCTTCTATCGTTCCCGAGCGTTCTAAACC

2341        .         .         .         .         .         .
   TTTTCTTTGATTTCGCAACTCAAGTTTATCAAATTTTCCCTTGTTTTCCTAGTAGAGTAA
   ============================================================
   AAAAGAAACTAAAGCGTTGAGTTCAAATAGTTTAAAAGGGAACAAAAGGATCATCTCATT

2401        .         .         .         .         .         .
   CTAGGAAGGTGTAGAAAAATATGGAAAATGAAAATAGAACAGATTGGACATTGCCTAGAA
   ============================================================
   GATCCTTCCACATCTTTTTATACCTTTTACTTTTATCTTGTCTAACCTGTAACGGATCTT

2461        .         .         .         .         .         .
   AGAATTTAAATCCAAAAACTAAACAGCCTTATAAGCGAGGTCGTAACTGGTGGATTGTTG
   ============================================================
   TCTTAAATTTAGGTTTTTGATTTGTCGGAATATTCGCTCCAGCATTGACCACCTAACAAC

2521        .         .         .         .         .         .
   TTTATCCTGAAAGTTTGCCAGAGAATTGGAAAGAGATTATTTCAACTGAACCTGTGGCAA
   ============================================================
   AAATAGGACTTTCAAACGGTCTCTTAACCTTTCTCTAATAAAGTTGACTTGGACACCGTT

2581        .         .         .         .         .         .
   TTAGTCCATTACATGATAAAGATGTTAATGCAGACGGAACAAAGAAAAAGCCGCATTATC
   ============================================================
   AATCAGGTAATGTACTATTTCTACAATTACGTCTGCCTTGTTTCTTTTTCGGCGTAATAG

2641        .         .         .         .         .         .
   ATATAGTTTTTAATTACAAGGGCAATAAATCATTTGAACAAATGGACGAAATGGCAAGAG
   ============================================================
   TATATCAAAAATTAATGTTCCCGTTATTTAGTAAACTTGTTTACCTGCTTTACCGTTCTC
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2701        .         .         .         .         .         .
   CTTTAAGGGCACCGATTCCTGAAAGAATAAGTGGTTTAACTGGTGCTGTTAGATATTTAA
   ============================================================
   GAAATTCCCGTGGCTAAGGACTTTCTTATTCACCAAATTGACCACGACAATCTATAAATT

2761        .         .         .         .         .         .
   CGCATATGGATAATCCTGAAAAGTATCAATACGATAATACAGAAATACAAGTGTTTGGTG
   ============================================================
   GCGTATACCTATTAGGACTTTTCATAGTTATGCTATTATGTCTTTATGTTCACAAACCAC

2821        .         .         .         .         .         .
   GATTTGACCTTGAAAGTTGTTTAGCGTTATCTACTGGTGATAAAAGACAAGCGTTAAAAG
   ============================================================
   CTAAACTGGAACTTTCAACAAATCGCAATAGATGACCACTATTTTCTGTTCGCAATTTTC

2881        .         .         .         .         .         .
   AAATGCTTGGTTTTATTTCGGATAACAATATTATGCATTTAAAAGATTTTGCTGATTATT
   ============================================================
   TTTACGAACCAAAATAAAGCCTATTGTTATAATACGTAAATTTTCTAAAACGACTAATAA

2941        .         .         .         .         .         .
   GTATGTCTGACCGAGCTCCTGCTGGTTGGTTCGAATTGCTAACAGAGAGGAATACTCTTT
   ============================================================
   CATACAGACTGGCTCGAGGACGACCAACCAAGCTTAACGATTGTCTCTCCTTATGAGAAA

3001        .         .         .         .         .         .
   TTATAAAAGAGTACATAAAATCGAATTGGCAAAAAGAAAACCAAGTTTATAAAGAGTGAG
   ============================================================
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3061        .         .         .         .         .         .
   GAATTAAATAAAAAATATGCCCCCTGCTGAAAAGCCAAGGGGGTTATTTTTTTGTGTTTT
   ============================================================
   CTTAATTTATTTTTTATACGGGGGACGACTTTTCGGTTCCCCCAATAAAAAAACACAAAA

3121        .         .         .         .         .         .
   AAGAGTTGGATTACATGAGCCAACTCTTTGTTTTTTGTATCAGCTAATTGCACCATAAGG
   ============================================================
   TTCTCAACCTAATGTACTCGGTTGAGAAACAAAAAACATAGTCGATTAACGTGGTATTCC

3181        .         .         .         .         .         .
   GTGCGCTGATACTCGGCTCAAAAGAGCCGAATAAGCCGTCTGCAAGACCCCTCGGCGAGC
   ============================================================
   CACGCGACTATGAGCCGAGTTTTCTCGGCTTATTCGGCAGACGTTCTGGGGAGCCGCTCG

3241        .         .         .         .         .         .
   CCACCTTTACGAAGTAAAGTATAGTGGGTTATACTTTGCATGGAAGCTGTCCCGAAGTTA
   ============================================================
   GGTGGAAATGCTTCATTTCATATCACCCAATATGAAACGTACCTTCGACAGGGCTTCAAT

3301        .         .         .         .         .         .
   GGAGTGTTTATATGTCATTTGTAGTGGCGAGAATGCAGAAGGTAAAATCAGGAAATTTAG
   ============================================================
   CCTCACAAATATACAGTAAACATCACCGCTCTTACGTCTTCCATTTTAGTCCTTTAAATC
```

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3361        .         .         .         .         .         .
   TTGGGGTAGGTAATCATAATCAGAGAAATACAGACAATCATTCCAACAAAGATATTGATG
   ============================================================
   AACCCCATCCATTAGTATTAGTCTCTTTATGTCTGTTAGTAAGGTTGTTTCTATAACTAC

3421        .         .         .         .         .         .
   TTGAACGGTCACATTTAAATTATGATTTGGTCAATCGGACAGAAAATTATAAACGAGATA
   ============================================================
   AACTTGCCAGTGTAAATTTAATACTAAACCAGTTAGCCTGTCTTTTAATATTTGCTCTAT

3481        .         .         .         .         .         .
   TTGAGCAATTTATTAACGACAACAAATCAAGTAGTCGTGCTGTCAGAAAAGATGCTGTAT
   ============================================================
   AACTCGTTAAATAATTGCTGTTGTTTAGTTCATCAGCACGACAGTCTTTTCTACGACATA

3541        .         .         .         .         .         .
   TAATAAACGAAAGAGTACTTTAAAGATTCTAAGCAAGTTACAACTGAACAGGTTAAAACG
   ============================================================
   ATTATTTGCTTTCTCATGAAATTTCTAAGATTCGTTCAATGTTGACTTGTCCAATTTTGC

3601        .         .         .         .         .         .
   CTTGTTAGTGGCGTTTTAGACAAAGTAAAAGAATTTGTAAAAGGTGGAGAATTTGAAAAA
   ============================================================
   GAACAATCACCGCAAAATCTGTTTCATTTTCTTAAACATTTTCCACCTCTTAAACTTTTT

3661        .         .         .         .         .         .
   ATTCATAGAAATGAAACTCAAACACGAGATAGAGATGAATTAAGTCGTTAGAAGCTCATA
   ============================================================
   TAAGTATCTTTACTTTGAGTTTGTGCTCTATCTCTACTTAATTCAGCAATCTTCGAGTAT

3721        .         .         .         .         .         .
   TTTGCGTTCTAAGACGTTTTAAATCAATTCGAGTATATTTACACGTGATTATTACTTTTC
   ============================================================
   AAACGCAAGATTCTGCAAAATTTAGTTAAGCTCATATAAATGTGCACTAATAATGAAAAG

3781        .         .         .         .         .         .
   GTCGGCATAAGCGTTTAAAATGGCTATAAATAATAAAGACAAGGGCGAACAACGTGAGCA
   ============================================================
   CAGCCGTATTCGCAAATTTTACCGATATTTATTATTTCTGTTCCCGCTTGTTGCACTCGT

3841        .         .         .         .         .         .
   ACGTTTACCCTTGTCTTTTTATTTTAGGCATTTAGCTTTTGCTGAGCGACGAAAAGTAAT
   ============================================================
   TGCAAATGGGAACAGAAAAATAAAATCCGTAAATCGAAAACGACTCGCTGCTTTTCATTA

3901        .         .         .         .         .         .
   TACGTGATAAAGCTACTTTTCAGAGGCATGATCAAACGCATTGCGTTTGTCAGGGGTGGG
   ============================================================
   ATGCACTATTTCGATGAAAAGTCTCCGTACTAGTTTGCGTAACGCAAACAGTCCCCACCC

3961        .         .         .         .         .         .
   CGGAAGCCCACGGTTTTTAAAAGGGTTTTTGCGGAGCAAAATACCCTTTTCTTCTTATCT
   ============================================================
   GCCTTCGGGTGCCAAAAATTTTCCCAAAAACGCCTCGTTTTATGGGAAAAGAAGAATAGA

4021        .4031
   TGATACTATTA
   ===========
   ACTATGATAAT
```